Antioxidant effects of flavonoids

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A Thesis submitted for the degree of Doctor of Philosophy

to

The Faculty of Medicine, University of Glasgow (1998)

from research conducted at the

Department of Human Nutrition, University of Glasgow

Glasgow Royal Infirmary, Glasgow, UK

Comet "Hale - Bopp" over Scotland, April 1997

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DEDICATION

To my parents, sisters and brother.

To my two flowers, my dear wife Rosa of whom I am proud for accompanying me for our PhD in the Department of Human Nutrition in Glasgow University, and also to our sweetheart daughter, Yasaman.

To the Ministry of Health and Medical Education of I.R. Iran for awarding scholarship for PhD in Human Nutrition.

To my supervisor Professor MEJ Lean, Department of Human Nutrition, University of Glasgow, Glasgow Royal Infirmary I wish to express my big thanks for his kind support and supervision.

SUMMARY

Flavonoids are polyphenolic compounds whose main dietary sources are fruits and vegetables. Epidemiological evidence has suggested that dietary flavonoids may protect against heart disease but biological effects have not hitherto been demonstrated directly in humans and there was no consistent evidence about the absorption of flavonoids. The studies performed for this thesis aimed to test antioxidant properties of flavonoids using an in vitro system, ex vivo tests on human tissue (lymphocytes) and in a dietary intervention.

The antioxidant effects of pre-treatment with flavonoids and vitamin C (as a positive control) in standardised concentrations (7.6, 23.2, 93 and 279.4 μmol/l), on oxygen-radical-generated DNA damage from hydrogen peroxide (100 μmol/l) in human lymphocytes were examined using the single-cell gel electrophoresis assay (SCGE assay or "comet assay"). Pre-treatment with all flavonoids and vitamin C produced dose-dependent reductions in oxidative DNA damage. At a concentration of 279 μmol/l, they were ranked in decreasing order of potency as follows: luteolin (9% of damage from unopposed hydrogen peroxide), myricetin (10%), quercetin (22%), kaempferol (32%), quercitrin (quercetin-3-L-rhamnoside) (45%), apigenin (59%), quercetin-3-glucoside (62%), rutin (quercetin-3β D-rutinoside), (83%) and vitamin C (78% of damage). The protection of vitamin C against DNA damage at this concentration was significantly less than that of all the flavonoids except apigenin, quercetin-3-glucoside and rutin. The protective effects of quercetin

and vitamin C at a concentration of 23.2 μmol/l were found to be additive (quercetin 71% of maximal DNA damage from unopposed hydrogen peroxide, vitamin C 83% , both in combination 62%). These data suggest that the free flavonoids are more protective than the conjugated flavonoids (e.g. quercetin versus its conjugate quercetin-3-glucoside, $p<0.001$). They are also consistent with the hypothesis that antioxidant activity of free flavonoids is related to the number of hydroxyl groups.

The next study involved detection of antioxidant activities of flavonoids in isolation and in human plasma in the trolox equivalent antioxidant capacity (TEAC) assays, to compare the antioxidant activities of some common flavonoids and vitamin C with that of trolox (a synthetic vitamin E) and to evaluate the effect of in vitro addition of flavonoids on the total antioxidant activity of human plasma. The antioxidant activities of 17 free and conjugated flavonoids and related polyphenolic compounds at the concentrations of 1mmol/l were tested in vitro and compared with vitamin C at the same concentrations in the TEAC assay. The total antioxidant activity of human plasma was measured using the same assay before and after adding rutin, quercetin and 100 μmol/l kaempferol in concentrations 10-100 μmol/l.

It was found that all flavonoids tested, except naringin, had more antioxidant activity than vitamin C ($p<0.05$) as measured in the standard TEAC assay. In addition, since Trolox, which is an analogue of vitamin E, at 1 mmol/l has a

TEAC of 1.0 (and this is the basis of calibration) then all flavonoids, (except narigin) also have greater antioxidant activity than vitamin E. Quercetin and rutin produced a dose-related increase in antioxidant capacity of normal human plasma. The addition of 50 μmol/l quercetin and 100 μmol/l quercetin, rutin and kaempferol significantly increased the total antioxidant capacity of human plasma ($p<0.001$). There was a strong positive correlation between the number of hydroxyl group of flavonoids and the antioxidant activity ($p<0.001$, $R = 0.86$). The flavonoid aglycones were more potent in their anti-free radical action than their corresponding glycosides ($p<0.05$).

Pilot studies were unable to show absorption of oral quercetin administration, so a dietary study was conducted to search for effects from food-derived flavonoids in diabetic patients (NIDDM). Non-insulin dependent diabetic patients were chosen because they have reduced antioxidant defences and suffer an excess of free-radical mediated diseases like coronary heart disease. Ten stable non-insulin dependent diabetic patients were treated for 2 weeks on a low flavonoid diet and for 2 weeks on the same diet supplemented with 110 or 76 mgs of flavonoids (mostly quercetin) provided by 400 g onions with ($n = 5$) or without ($n = 5$) tomato ketchup and 6 cups of tea daily, in random order.

Fasting plasma of flavonoid concentrations were undetectable (< 1 ng/ml) in 7/10 subjects, mean 5.6 ± 2.9 ng/ml on the low flavonoid diet. This was increased to 52.2 ± 12.4 ng/ml on onion and tea supplemented diet containing

76.3 mg flavonoids daily ($p<0.001$), almost all from quercetin. Fasting plasma flavonoid rose to 87.3 $\pm$ 26.7 mg/l on the onion, tomato ketchup and tea supplemented diet which contained 110 mg/day flavonoids. Urine collections revealed a similar 13-fold increase in flavonoid excretion on the supplemented diets, and the fasting plasma and 24 hour urinary flavonoids were highly correlated ($r = 0.75$).

Oxidative damage to lymphocyte DNA on an arbitrary scale 0 to 400 units was 220 $\pm$ 12 on the low flavonoid diet and 192 $\pm$ 14 on the high flavonoid diets ($p=0.037$). This increased antioxidant activity on the high flavonoid diet was not accounted for by any change in measurements of diabetic control (fasting plasma glucose or fructosamine), nor by any change in plasma measurements of known antioxidants including vitamin C, carotenoids, tocopherols, urate, albumin, bilirubin. Other phenolics, e.g. catechins were not measured.

Analysis of the plasma, urinary and dietary flavonoids indicated that dietary consumption can be predicted by 24 hour urine ($r^2 = 0.75$) or fasting plasma concentration ($r^2 = 0.51$). The habitual (baseline) diets of these diabetic patients contained 20-80 mg/day, mean 33 mg/day.

The main conclusions of this thesis are:

1. There is a potent antioxidant action of dietary flavonoids demonstrated by the comet assay, of potential importance in protection against cardiovascular disease and cancer.
2. The antioxidant capacities of most major dietary flavonoids are greater than vitamin C.
3. Results from the comet assay and TEAC show reasonable agreement in ranking.
4. Antioxidant activities of free flavonoids are more than the conjugated flavonoids.
5. There were a strong positive correlation between the number of hydroxyl group of flavonoids and the antioxidant activity
6. Dietary flavonoids are absorbed and the fasting plasma concentration can be increased 12 fold by a simple and palatable food supplement.
7. Supplementation with onions, tomato ketchup and tea lead to protection of lymphocytes against free radical damage (H_2O_2), a biological effect of potential medical importance possibly attributable to the absorption of dietary flavonoids.
8. Dietary flavonoids intake (and specifically quercetin) can be estimated with reasonable accuracy from 24 hour urinary flavonoid excretion or fasting plasma concentration.
9. The range of dietary flavonoid consumption in ten NIDDM patients was estimated at 20-80 mg/day from their normal diets. On the basis of results in

this thesis, dietary difference within this range would influence tissue antioxidant status.

DECLARATION AND EXTENT OF COLLABORATION

The present thesis has used several different experiment designs and were carried out by myself in the laboratory of the University Department of Human Nutrition, Glasgow Royal Infirmary, under the principal supervision of Professor MEJ Lean, and with invaluable teaching and guidance from a number of senior colleagues. I personally designed all the experiments and conducted all the ordering and preparing of chemicals and solutions for in vitro, in vivo and ex vivo experiments and analysed them myself, except where acknowledged. To develop the SCGE assay (Chapter 1) I used my own capillary venous blood (many times). The extent of collaborations and my personal input to the research are indicated in each Chapter. Routine biochemical assays outlined in Chapter 2 were all conducted by staff in the Department of Pathological Biochemistry, Glasgow Royal Infirmary. Dietetic supervision and diet analyses were conducted by Ms Irene Kelly, SRD, Department of Human Nutrition.

Mostafa Noroozi [redacted]

.....[redacted].....

I certify that the work reported in this thesis has been performed by MOSTAFA NOROOZI, and that during the period of study he has fulfilled the conditions of the ordinances and regulations governing the Degree of Doctor of Philosophy.

Professor MEJ Lean [redacted]

.....[redacted]....

ABBREVIATIONS

LDL = Low density lipoprotein

HDL = High density lipoprotein

VLDL = Very low density lipoprotein

CHD = Coronary heart disease

EC = Epicatechin

EGCG = Epigallocatechin gallate

ECG = Epicatechin gallate

8-oxodG = 8-oxo-7,8-dihydro-2'-deoxyguanosin

SOD = Superoxide dismutase

GPX = Glutathione peroxidase

PBS = Phosphate-buffered-saline

OFRs = Oxygen free radicals

ROS = Reactive oxygen species

PUFAs = Polyunsaturated fatty acids

APO-B = Apolipoprotein B

NIDDM = Non-insulin dependent diabetes mellitus (Type 2)

IDDM = Insulin dpendent diabetes mellitus (Type 1)

Cu-Zn SOD = Copper and zinc containing superoxide dismutase (largely in the cytosol)

Mn SOD = Magenese containing superoxide dismutase (active site in the mitocondria)

CAT = Catalase

EDTA = Ethylenediaminetetracetic acid

PMNs = Polymorphonuclear leukocytes

$^{\bullet}O_2^{-}$ = Superoxide radical

WBC = White blood cells

AA = Ascorbic acid

DHA = Dehydro ascorbic acid

GSH = Reduced glutation

GR = Glutation reductase

TBARS = Thiobarbituric acid reactive substances

TRAP = Total radical-trapping antioxidant parameter

($OH^{\bullet}$) = Hydroxyl radical

IGT = Impaired glucose tolerance

AP = Alkaline phosphatase

GPT = Glutamic-pyruvic transaminase

GOT = Glutamic-oxalecetic transaminase

LDH = Lactic dehydrogenase

TOOS = N-ethyl-N-(2-hydroxy-3-sulfopropyl)-3-methylaniline

CHAPTER 1: Literature review and background

1.1 Free radical and antioxidants in health and disease

1.1.1 What is a free radical and why it is important

A free radical is an atom or molecule that contains one or more unpaired electrons (Halliwell 1994) or a chemical species with unpaired electron which can be formed by loss or adding a single electron to a normal molecule or by the cleavage of a covalent bond of a normal molecule, such that each fragment contains one of the paired electrons (Cheeseman & Slater 1993). The hydrogen radical contains one proton and electron, and is the simplest free radical (Halliwell 1994) and oxygen is the most important free radical in biological systems (Cheeseman & Salter 1993). The half life of free radicals is short and they do not travel far because they are highly reactive, often reacting at their site of formation (Halliwell 1994). The main pathological importance of free radicals is their destructive action against three molecular forms in the body: DNA, proteins and lipids. They release high energy, and may initiate chain reactions by producing further free radicals. Their oxidative actions against DNA, proteins and lipids are often permanent.

1.1.2 The source of free radicals

Free radicals produced as a normal consequence of aerobic metabolism and for microbial killing by neutrophils (Halliwell et al 1994). Within the human body, some conditions promote generation of hydroxyl radical ($OH^{\bullet}$) and superoxide ($O_2^{\bullet-}$). Approximately 1-3% of oxygen we breath is used to make superoxide by activated phagocytes cells (neutrophils, monocytes, macrophages and

eosinophils) for killing the foreign organisms (Catapano et al 1997). Nitric oxide ($NO^{\bullet}$) is another physiological free radical which is produced by the enzyme nitric oxide synthase from L-arginine in phagocytes and by vascular endothelium where it acts as a relaxing factor (Moncada et al 1993).

Some free radicals are formed in the human body both by accidents of chemistry and for useful metabolic purposes (Halliwell 1996). Free radicals are generated by normal metabolic processes e.g. reduction of oxygen to water by mitochondrial electron transport chain. From all oxygen used in metabolism, 1-5% escapes as free radical intermediates (Neville et al 1996). Loss of a single electron of oxygen produces the superoxide free radical anion ($O^{-\bullet}_2$) or reduction of two-electron of oxygen produce (H_2O_2) and H_2O_2 can also be produced by reaction of two superoxide together with ($2O^{-\bullet}_2 + 2H^+ \rightarrow H_2O_2 + O_2$) (Cheeseman & Slater 1993). Ionizing gamma rays, radiation ultraviolet can split water in the human body to generate ($OH^{\bullet}$) (Halliwell 1994). **Table 1.4** presents a summary of generation of free radicals. Excess exposure to free radicals, whether endogenously produced or derived from environment, leads to oxidative stress and potential severe damage through an oxidative cascade affecting lipids, proteins or nucleic acids

1.1.3 Effects of free radical on lipids

Lipid peroxidation of cell membranes begins with the abstraction of (H) atom from a (CH3) group, and most often occurs in the proximity of a double bond. The free radical then re-arranges into conjugated diene, that can react with an oxygen molecule to form a peroxyl radical ($ROO^{\cdot}$). Peroxyl radicals can be changed to form lipid hydroxides (ROOH) by removing (H) from other lipid molecules, and setting in motion a chain reaction that can oxidase unsaturated fatty acids in the membrane. Lipid hydroperoxides (ROOH) can react with transition metals (Fe, Cu) to produce hydroperoxy ($ROO^{\cdot}$) or alkoxy ($RO^{\cdot}$) radicals, they can react with Fe^{2+} to form Fe^{3+}, hydroxide ion and alkoxyl radical ($RO^{\cdot}$) radicals. This is similar to the Fenton reaction ($ROOH + Fe^{2+} \rightarrow Fe^{3+} + OH^{-} + RO^{\cdot}$) (Hunt 1993).

Lipid peroxidation commonly starts in polyunsaturated fatty acids (PUFA) in LDL, and in surface phospholipids, and the process gives rise to a wide variety of active molecules such as oxidised sterols, modified phospholipids, products of oxidised fatty acids (Malonyldialdehyde and hydroxynonenal) that can react with Lysine in apolipoprotein B (APo-B) and produce lysophosphatidylcholine, can which if released from the modified LDL causes damage to artery walls (Witztum 1994). Oxidised LDL is not recognised by the normal LDL receptors, and can only be removed from the circulation via the scavenger pathway. Accumulation of oxidised LDL in macrophages to form "foam cells" is considered an important factor in the origin of coronary heart disease (CHD).

Superoxide has a role in the oxidation of low density lipoprotein (LDL) and also (NO) may act both as an oxidant and antioxidant and by its ability to form peroxynitrite with superoxide radicals, it would be expected to oxidise LDL (Catapano et al 1997). Therefore antioxidants could prevent peroxidation of lipid by free radicals and protect from vascular disease.

Excess generation of reactive oxygen species (ROS) in vivo occur by the action of catalytic ions (Fe, Cu) in the cell membrane which can induce lipid peroxidation (Halliwell 1996). Oxidation of PUFA generates fatty acid radicals ($L^{\cdot}$) which by adding O_2 produce fatty acid peroxyl radicals ($LOO^{\cdot}$), which in turn can be carriers of the chain-reaction to oxidise further (PUFA) molecules, finally producing lipid hydroperoxides (LOOH), more radical species and aldehydes (Cheeseman & Slater 1993).

(1) $LH + R^{\cdot} \rightarrow L^{\cdot} + RH$

(2) $L^{\cdot} + O_2 \rightarrow LOO^{\cdot}$

(3) $LOO^{\cdot} + L^{\cdot}H \rightarrow LOOH + L^{\cdot}$

(4) $LOOH \rightarrow LO^{\cdot}, LOO^{\cdot}$, aldehydes

Oxidised fatty acids in LDL can react with lysine in APo-B and produce a new oxidised LDL which is then recognised by the macrophages scavenger receptors (Witztum 1994). PUFA appear more susceptible to free radical attack than proteins and nucleic acids (Cheeseman & Slater 1993).

1.1.4 Effect of free radical damage on protein and DNA

Peroxynitrite, formed from two free radical molecules (superoxide and nitric oxide) at physiological pH, directly damages proteins (Halliwell 1994).

Hydroxyl radicals induce DNA damage by interaction of hydrogen peroxide and superoxide with transition metals (Guyton & Kensler 1993). Both Fe and Cu ions can promote DNA damage by active oxygen species in vivo (Aruoma et al 1991). Incubation cells with H_2O_2 induces DNA damage which can be inhibited by catalase, while (SOD) does not usually inhibit this process much (implying possibly that DNA damage is not mediated by $O_2^{\cdot}$). It seems $OH^{\cdot}$, generated from H_2O_2, crosses biological membranes, and can diffuse to the nucleus (Halliwell & Gutteridge 1990). Prutz et al (1990) suggested that metal ions are always present bound to the DNA, for example Cu is in the chromosomes, and are very effective in promoting H_2O_2 dependent damage to chromatin DNA or isolated DNA. Another possibility is that interacellular free Ca^{++} is released within the cells as a result of oxidative stress, and bind to the DNA to make it a target for oxidative damage (Halliwell & Aruoma 1992).

Oxidative stress causes rises in intracellular free Ca^{++} which fragment DNA by activating Ca^{++} dependent nuclease (Orrentus et al 1989). Both mechanisms, DNA damage by $OH^{\cdot}$ or by activation of Ca^{++}-dependent nuclease, could take place (Halliwell & Aruoma 1992). Chelating agents can protect cells against DNA damage and other effects of oxidative stress (Mello-Fiho et al 1984) by

removing metal ions from the vicinity of DNA, so that any $OH^{\cdot}$ generated no longer attacks molecules (Halliwell & Gutteridge 1990).

Comparing reaction mixtures of H_2O_2 and $O^{\cdot\cdot}_2$ showed that Cu mediates much more damage to DNA bases by $OH^{\cdot}$ generated from H_2O_2 than Fe (Aruoma et al 1991) because Cu reacts faster than Fe with H_2O_2 to form $OH^{\cdot}$ (Halliwell & Gutteridge 1989). The radical $OH^{\cdot}$ is an oxidant from normal metabolism and endogenous processes leading to significant DNA damage which is likely to involve oxidation, methylation, deamination and depurination (Ames & Shigenaga 1992).

$$O_2 \xrightarrow{e^-} O_2^{\cdot-} \xrightarrow{e^-} H_2O_2 \xrightarrow{e^-} OH^{\cdot} \xrightarrow{e^-} H_2O_2$$

The genotoxic effects of hydrogen peroxide, a common end product of many types of oxidative stresses, have been studied (Martins et al 1990). Hydroxyl radical generated from H_2O_2 produces DNA strand breaks. In the Fenton reaction model, DNA-bound Fe^{2+} reacts with H_2O_2 to generate OH radical, and this reactive site-generated radical will then attack DNA to produce damage (Meneghine et al 1988).

Hydroxyl radicals can attack the purine and pyrimidine bases and cause mutations e.g. guanine is converted into 8-hydroxyguanine (Halliwell 1994) or can attack both the deoxyribose sugar and the purine and pyrimidine bases,

forming a wide range of products. At least 18 products of oxidative damage to the DNA bases, have been detected, for example: 8-hydroxyguanine, 8-hydroxyadenine, 2-hydroxyadenine, 5-hydroxycytosine, 5-hydroxyuracil and 5, 6-dihydroxyuracil. The amount of these OH-derived products in urine or DNA extracted from tissue shows attack by ($OH^{\bullet}$) and unrepaired DNA damage (Halliwell 1996).

The compound 8-oxod G (8-oxo-7, 8-dihydro-2'-deoxyguanosine) is potentially the most mutagenic compound induced in DNA by reactive oxygen species (Olinski et al 1992). After DNA repair by excision the resulting product, 8-oxodG, is excreted unchanged into the urine (independently of diet) and is a good biomarker of oxidative DNA damage in the whole body (Shigenaga et al 1989). Verhagen et al (1995) using the 8-oxodG method found a reduction of oxidative DNA damage in humans with consumption of 300 g/day of cooked brussel sprouts during 3 weeks in 10 subjects. They concluded that the consumption of cruciferous vegetables (cabbage, broccoli, brussel sprouts) may decrease cancer risk.

Oxidative DNA damage is considered a pathogenic event in many cancers (Ames and Gold 1991, Cenitti 1994), therefore decreases in the rate of oxidative DNA damage may indicate a reduced risk of cancer. Antioxidant vitamins, flavonoids, glucosinolates, organosulfur compounds) have been claimed to have antimutagenic or anticarcinogenic potential (Verhagen et al 1995). Oxidative

damage to DNA by free radical mechanisms is a main cause of cancer in humans. However the process may not necessarily lead to cancer, as low levels of damage may be repaired with a minimal risk of error (Breimer 1991). Therefore it may not be necessary for antioxidants to stop free-radical production, but to restrict it to a certain level.

1.1.5 Antioxidant defence against free radicals

There are several interacting systems in the body which neutralise free radicals, or prevent their production, and stop free-radical chain reactions. They appear to operate in a cascade. Some antioxidant defence against free radical present in **(Table 1.5)**.

Superoxide dismutase (SOD) is a free radical scavenger (Cheeseman & Slater 1993) which converts superoxide to hydrogen peroxide in the mitochondria and cytosol ($2O_2^{\cdot-} + 2H^+ \rightarrow H_2O_2 + O_2$). Glutathione peroxidase is a major enzyme that removes hydrogen peroxide generated by SOD in cytosol and mitochondria (Chance et al 1979), by changing to oxidised form (GSSG):
($2GSH + H_2O_2 \rightarrow GSSG + 2H_2O$). Catalase also remove H_2O_2 in peroxisomes in most tissues (Halliwell 1994).

Diets rich in fruits, nuts, grains and vegetables seem to be protective against several human diseases (Catapano et al 1997). The assumption is made that common dietary and endogenous antioxidants operate at different sites. Vitamin

C (as a cytosolic antioxidant), vitamin E (as membrane antioxidant), GPX (as a membrane, cytolic and plasma antioxidant), SOD (as a mitochondial, cytosolic and extracellular antioxidant), catalase, carotenoids and ubiquinones are believed to act together to protect both cytosol and membranes against free radical attack (Neville et al 1996). Catalase and GPX are enzymes whose role is to decompose peroxides safely, mainly located in peroxisomes and cytosol. Antioxidant proteins (enzyme and non-enzyme), lipid and water soluble antioxidants in human plasma and urine and also some flavonoids and polyphenols antioxidants are presented in **Table 4.1.**

The most important lipid chain breaking antioxidant is α-tocopherol. It works by intercepting lipid peroxyl radicals ($LOO^{\cdot}$) to form a tocopheroxyl radical which is insufficiently reactive to initiate lipid peroxidation itself (Stocker et al 1991).

$LOO^{\cdot}$ + α-tocopherol-OH → LOOH + α-tocopherol – $O^{\cdot}$

The tocopherol radicals (located in membranes and lipids) is then restored to α-tocopherol through the action of ascorbic acid and other water soluble and circulating antioxidants at the surface of membranes and lipoproteins (Mukai et al 1993).

($\alpha TH + LOO^{\cdot} \rightarrow \alpha T^{\cdot} + LOOH$)

Another part of natural antioxidant defence is the repair of oxidative damaged nucleic acids by specific enzymes, oxidised protein by proteolytic systems and the removal of oxidised membrane lipids removed by lipases, peroxidases and acyltransferases (Cheeseman & Slater 1993).

Haemopexin and haptoglobin bind free heme and heme proteins to reduce their ability to catalyse free-radical damage. Albumin which has antioxidant properties because it contains a sulphydryl group, can scavenge several radicals and binds copper ions in plasma (Halliwell 1990).

1.1.6 Free radicals in human disease

Inflammatory reaction: In the normal inflammatory reaction, leucocytes, neutrophils and macrophages possess NADPH oxidase and release superoxide by one electron reduction of oxygen. Reactive oxygen species (ROS) are normally created by leucocytes to kill ingested or extracellular bacteria (Neville et al 1996).

Ischaemia: Xanthine oxidase acts as a source of oxygen free radicals (OFRs) and superoxide ($O_2^{\bullet -}$). These products play a major role in generating tissue damage and endothelial dysfunction (Neville et al 1996). Animal studies suggest that myocardial ischaemia reperfusion causes oxidative stress, referred to by Oostenbrug et al (1997).

Skin cancer: Exposure to ultraviolet radiation and the effects of sunburn generate free radicals in the skin. The decrease in the protective ozone layer has relation with increased level of ultraviolet radiation and lead to risk of skin cancer (Neville et al 1996).

Granulomatous disease: Children with chronic granulomatous disease die from bacterial infections because they have genetic deficiency which prevents their leucocytes from generating OFRs (Neville et al 1996), needed to set up normal inflammatory responses to infection.

Hypertension: Free radicals are believed to play a role in the control of blood pressure (Kumar & Das 1997). Excess vascular $O_2^{\bullet -}$ could lead to hypertension (Nakazone et al 1991) because of imbalance between ($NO^{\bullet}$) as a relaxor of vessel walls and ($O_2^{\bullet -}$) as a vascoconstrictor by removing ($NO^{\bullet}$) (Laurindo et al 1991). Therefore over production of superoxide might be one cause of hypertension (Halliwell 1994).

Atherosclerosis: Free radicals can induce oxidative damage and have been implicated in diseases such as atherosclerosis (Witztum et al 1994). PUFA in membranes can be oxidised by free radicals producing lipid hydroperoxides (directly) and reactive aldehydes (indirectly). Lipid peroxidation may be involved in the pathogenesis of atherosclerosis. Oxidised lipoproteins (LDL) cannot be processed by the normal receptors, and tend to accumulate in

macrophages through scavenger receptors. These accumulate within vessel walls as foam cells, the precursors to fatty streaks (Cheeseman & Slater 1993). Diets rich in fruits and vegetables are protective against cardiovascular disease, and their antioxidant content is a possible mechanism (WHO 1990).

Lung related disease: Evidence of oxidative reactions is often associated with fibrogenesis ocurring in liver and lung (Poli & Parola, 1997). Lung fibrosis and cancer caused by dusts such as silicates and asbestos may be partly mediated by ROS (Janssen et al 1993). Peroxynitrite (combined superoxide and nitric oxide) ($ONOO^-$) is a strong oxidant that may contribute to lung injury. An increase in ROS has been shown in asthma and cystic fibrosis (Cross et al 1994). Lungs are vulnerable to infection (pneumonia) if protecting ROS are not released by leukocytes as part of the normal inflammatory response.

Neurodegenerative disease: In both Parkinson's disease and Alzheimers disease iron increases in substantia nigra and cortical regions and there is also reduced GSH. In Parkinson's disease hydroxylated guanine (8-oxod G) a product of free radical attack on guanine in DNA, is raised. However SOD (Cu/Zn and Mn) are above normal which may be an adaptative change to increased free radical load, (Jenner 1994). Deficiency of vitamin E, specifically in abetalipoproteinaemia, results in optic atrophy and cerebral degeneration which are believed to be the result of unopposed free radical activity.

Premature babies: Exposure to high concentrations of oxygen before adequate antioxidant defences are developed, produce toxic reactions and tissue injury (Neville et al 1996).

1.1.7 Antioxidant intervention studies

Most analyses have sought mechanisms based on antioxidants routinely included in nutrient data bases, and on the basis of this type of research, intervention studies using vitamin C, vitamin E and beta carotene have been mounted. The potential for error in this approach was demonstrated by studies (The Alpha-tocopherol group 1994) which showed no benefit, indeed potential harm, from isolated beta carotene supplementation. On the other hand vitamin E supplementation seems to protect against heart disease, and vitamin C and E against cataract. Foods which contain the conventional antioxidant vitamins are often rich in phenolecs which may be bioactive. On this basis, food-based interventions may be safer and more successful than using isolated vitamins or other compounds.

1.1.8 Free radicals and antioxidants in diabetes

Diabetes mellitus is a metabolic disorder characterized by high blood glucose, polyuria, thirst, hunger, emaciation and weakness. Underlying the disease is either deficiency (Type I) or resistance (Type II) to insulin. Type I patients are characteristically young and lean, totally dependent on exogenous insulin, while Type II are usually obese and older and can be treated with dietary changes,

exercise and oral medication (Oberley 1988). The main clinical hazards of Type I diabetes are microvascular diseases, and in Type II diabetes an acceleration of ischaemic heart disease and cerebrovascular disease. Colditz et al (1992) have produced evidence from the Boston Nurses Study that fruit and vegetables protect against the development of diabetes. Antioxidants provide one possible mechanism.

Diabetic patients, both Type I and Type II, have abnormal antioxidant status, with auto-oxidation of glucose and excess glycosylated protein (Jones et al 1985). Oxidative stress leads to tissue damage, increased reactive oxygen species, inactivation of proteins, fragmentation of DNA and tissue degeneration in diabetes mellitus (Loven et al 1985). There is already abnormal antioxidant status in the pre-diabetic state of impaired glucose tolerance (IGT) and this may relate to the high coronary heart disease risk in IGT (Vijayalingan et al, 1996). Sinclair et al reported that there is a negative correlation between serum ascorbic acid and fructosamine concentration in diabetic patients with complications (Sinclair et al, 1992). They also found a low concentration of plasma ascorbate in patients with type 2 diabetes mellitus consuming adequate dietary vitamin C, and suggested that this implies increased utilisation of vitamin C to inactivate free radicals. Decreased lipid peroxidation and improved antioxidant status may be one mechanism by which dietary treatment contributes to the prevention of diabetic complications (Armstrong et al 1996). Increased free radical activity in diabetes mellitus may contribute to higher prevalence and mortality from

macrovascular disease in diabetic patients (MacRury et al 1993). Peripheral vascular disease, renal failure, coronary heart disease, cataract blindness, infections and artherosclerosis in diabetic patients all involve processes in which oxygen free radicals have been implicated (Oberley 1988).

A general increase in free radical activity in diabetes might be expected to result in increased cancer risk. According to recent research, IDDM and NIDDM patients demonstrate greater oxidative DNA damage with increased generation of reactive oxygen species than controls (Dandona et al, 1996). These findings are consistent with previous studies which have shown diabetes to be a risk factor for cancer of the uterine corpus and a positive association with prior diagnosis of diabetes was also noted for kidney cancer and non melanoma skin cancer in females (O'Mara et al 1985). In general, however, diabetes is not a major risk factor for cancer, and this argues for free radicals having a relatively lesser impact on cancer than on vascular disease, at least in this condition.

Defective serum antioxidant status contributes to the increased oxidative stress in diabetic patients. (Asayama et al 1993). Ceriello and co-workers (1997) suggested, decreased total radical-trapping antioxidant parameter (TRAP) levels in NIDDM patients is caused by lower antioxidant defences. In diabetic patients changes in superoxide dismutase (SOD) and catalase (CAT) activity, GSH metabolism, vitamin C and E level, and in lipid peroxides have been observed.

These topics are summarised below:

A: Superoxide dismutase

Matkovics (1982) found a 97% reduction in erythrocyte Cu-Zn SOD activity with increased glutathione peroxidase in NIDDM subjects maintained on oral hypoglycaemic agents. A glycosylated form of Cu-Zn SOD, which had a lower enzymatic activity, was increased in the erythrocytes of diabetic patients (Arai et al 1987). $O^{\cdot}_2^{-}$ in polymorphonuclears (PMNs) from diabetic patients were higher than normal (Nath et al 1984) which attributed to the decrease in the SOD activity in mitochondrial and cytoplasma. Treated diabetic patients with insulin showed increasing levels of SOD (Oberley 1988), and high level of glucose is not responsible for dysfunction of white blood cells (Pickering et al 1982). Erythrocyte SOD of NIDDM cases (n=467) was lower than normal (n=180) (Sundaram et al 1996), but the results of Kaji et al (1985) showed no changes in erythrocytes SOD activity in 60 NIDDM compared with 71 healthy control women. Although the literature is not entirely consistent it is concluded that SOD is reduced in diabetic patients.

B: Vitamins

Measurement of vitamin C and E in cases of NIDDM compared with healthy controls showed low levels of these scavenger vitamins (50% decrease for vitamin C), but no change in vitamin A (Sundaram et al 1996). Ascorbic acid (AA) and dehydroascorbic acid (DHA) in NIDDM patients showed low plasma levels compared with control (Sinclair et al 1994) which appeared to be a

consequence of the disease itself and not due to inadequate dietary intake of vitamin C. Low AA in NIDDM patients (despite high intake of vitamin C) may result from increased oxidative stress (Sinclair et al 1991). DHA/AA ratio of elderly diabetic patients (n=50) was higher than in controls (n=40) which implies that there had been increased utlisation (Sinclair et al 1992). Plasma α-tocopherol of NIDDM patients (n=23) observed by Vatassery et al (1983) were 31% higher than in a control group (n=30). This result is probably explained by the elevated plasma lipoproteins in the diabetic subjects, tocopherol being transported in lipoproteins.

C: GPX and GSH

Reduced glutathione (GSH) content of erythrocytes NIDDM patients was decreased (25%) compared with controls, but GPX was increased by 25% in NIDDM without complications, and by 85% in NIDDM with multiple complications, when compared with the controls. This may be an adaptive mechanism developed to deal with the increased generation of free radicals (Sundaram et al 1996). Kaji et al (1985) and Sinclair et al (1992) found no changes in GPX activity in NIDDM subjects. However, Uzel and Co workers (1987) found it decreased and Matkovics et al (1982) found increased GPX activity. On balance it is concluded that there may be increased demand for (GPX and therefore selenium) in diabetic patients, at least in certain situations.

D: Lipid peroxidation

Plasma and erythrocytes lipid peroxidation products (TBARS) in NIDDM patients showed a significant elevation (by 80%) in patients within the first 2 years of diagnosis compared with healthy subjects (n=180). Inactivation or inhibition of antioxidant enzymes by glycosylation in poorly controlled diabetes mellitus may give rise to increased lipid peroxidation (Sundaram et al 1996). Sinclair et al (1992) examined TBA reactivity of 50 NIDDM patients and control(n= 40) subjects and found no significant differences. But Hayaishi and Shimizu (1982) found 91% elevated TBA in NIDDM with angiopathy relative to controls and they suggested increased TBA-reactive material originated from intima of the blood vessel and might be related to the development of atherosclerosis. Well controlled NIDDM patients were unchanged in serum (TBA) reactive material, while there was an 61% increase in adult subjects with poorly controlled diabetes (Sato et al 1979). The evidence is therefore reasonably certain that lipid peroxidation is increased in diabetic patients, in keeping with their increased atheroma and vascular disease.

E: Catalase

Kaji et al (1985) found no change in erythrocytes CAT activity of either NIDDM or IDDM subjects. However, Sundaram and coworkers (1996) found decreased catalase in NIDDM (n=467) compared to controls (n=180). Also Matkovics et al (1982) presented data to show a lower CAT in diabetic patients. There is

therefore no clear abnormality of catalase in diabetic patients. Wataa et al (1986) showed increase in CAT activity in erythrocytes of diabetic children.

F: Treatment of diabetic patients with antioxidants

Treatment with antioxidants may thus be predicted to prevent or delay abnormalities associated with diabetes mellitus (Dandona et al 1996). Taken as a whole, the evidence discussed above points strongly to an increase in free radical mediated processes, and a reduction in several antioxidant defences in patients with diabetes. These processes are involved in long term tissue damage and diabetic complications. There appears to be increased demand for exogenous antioxidants and a case can be made for examining treatment with antioxidants. At present there is surprisingly little published information on administration of vitamin C, and/or vitamin E in diabetic patients to indicate long term benefit. Jacques et al (1997) have shown protection against cataract with vitamin C supplementation, and against CHD with vitamin E supplementation, but these studies did not include diabetic individuals. High dose vitamin E appears to increase insulin secretion (Paolisso et al 1993) but this is not a physiological mecahnism.

1.2 Chemical structure of flavonoids

Flavonoids are of the more numerous groups of natural products. Over 4000 different naturally occuring flavonoids have been described (Middleton & Kndaswami 1994) and the number of flavonoids is still growing. Subclasses of flavonoids and classification are based on variations in the heterocyclic ring shown in six groups: flavones, flavonols, flavanones, catechins, anthocyanidins and isoflavones **(Figure 1.1)** (Hollman et al 1997). The polyphenol bases have multiple hydroxyl groups, which are variably conjugated, giving rise to the huge number of specific compounds.

Plant flavonoids are a wide range of low-molecular-weight secondary metabolites (Rhodes 1996). The structural basis of flavonoids includes two benzene rings (A and B) combined by mediation of the oxygen containing ring C **(Figure 1.1)** (Kühnau et al 1976). The flavonoids are a related group of compounds based on the flavone nucleus, biosynthetically derived from phenylalanine and malonate (Rhodes 1996).

Flavonoids in plant materials can be in the form of glycosides. The most common glycosides of quercetin occuring in food plants appear to be the 3-glucoside (iso quercetin), 3-rhamnoside (quercitrin), 3-rhamnoside-galactoside (bioquercetin) and 3-rutinoside (rutin) (Brown 1980).

In the flavonol and anthocyanin series, conjugates with sugars and organic acid at 3 position in the C ring are common and sometimes at position 5 and 7 in the

A ring. Glucose, galactose, rhamnose, rutinose, malonate, acetate, and caffeic acid are linked with flavonoids and significantly increases the molecular weight of the flavonoids (Rhodes 1996).

Flavonoids generally occur as glycosides with sugars bound at the C3 position in our diet (Hertog et al 1993b). Flavonoids consist mainly of anthocyanidins, flavones, flavonols, catechins and flavonones (Herrmann 1988 and Hollman 1995). Anthocyanins are the pigments responsible for the red and blue colours of many fruit and vegetables (Rhodes 1996). Flavonoid compounds range from the yellow and colourless flavanones in citrus fruit to the red and blue anthocyanins in berries (Kühnau 1976). Two benzene rings (A and B) are linked to a heterocyclic ring (C) of flavonoids. This basic structure, particularly in C ring (C3) allows links with diferent substances and produce variations of flavonoids e.g. quercetin has been described with more than 179 different types of glycosides (Hertog et al 1996). Tannins are based on the flavon-3-ol structure. Flavonoids structure are based on the flavone nucleus, biosynthetically derived from phenylalanine and malonate. The various classes of flavonoids differ in the pattern of substitution of the A, B and C rings. From a dietary point of view, the most important classes of flavonoids are the flavones, flavonols, catechins, anthocyanins. Whereas these flavonoids are found in nearly all fruit and vegetables, the isoflavones found in legumes have activity as phyto-oestrogens in which the aromatic B ring is attached to position 3 of the C ring rather than position 2 as in the flavonoid series (**Figure 1.1**) (Rhodes 1996).

Optimum antioxidant activity of flavonoids is associated with multiple phenolic groups (3' and 4' hydroxy groups), a double bond in C2-C3, a carbonyl group at C4 and free C3 and C5 hydroxy groups (Roback et al 1988a; Rice-Evans et al 1996) **(Figure 1.1)**. Glycosylation is believed to reduce antioxidant activity (Shahidi 1992).

From the nutritional point of view,the physiochemical properties of flavonoids (flavonones, flavonols, flavones and even most anthocyanins) are relatively stable substances resistant against heat, oxygen, dryness and moderate degrees of acidity, but they are sensitive to light in in vitro situations). Therefore during the preparation in the kitchen or food processing, flavonoids are not greatly damaged (Kühnau et al 1976).

1.3 Biological function of flavonoids

1.3.1 Essential food factors

Claims of vitamin like activity for citrus groups of flavonoids have been made for several decades. They are marketed as "Bioflavonoids" in many countries. However, no conclusive data have been obtained to show that the flavonoids are essential food elements in humans. However some observations point to quite a fundamental and essential role of flavonoids in some insects (butterflies and silk worm larvae) which could be defined as 'vitamin like'. There are strong

indications that in lower stages of animal life flavonoids have indeed the character of essential food components, e.g. insects (Kühnau 1976). There is certainly evidence for "vitamin-like" actions. After oral supplementation with flavonols (catechins) the storage and antiscorbutic potency of vitamin C is increased in guinea pig organs, primarly in adrenals, kidneys, spleen and liver (Hughes and Jones 1971).

In the past a number of terms have been used for flavonoids e.g. "pseudovitamins" by Herbert (1988) and "semi essential food factors" or 'vitamin like' by Kühnau (1976), "vitamin P" for decreased permeability of the blood capillaries by "bioflavonols" (Rusznyk & Szent-György 1936), "vitamin C2" for protection of vitamin C from oxidation by flavonoids (Singleton 1981). In 1950 the term 'vitamin P' was discontinued and the term bioflavonols has replaced it (Herbert 1988).

1.3.2 Metal-chelating capacity of flavonoids

Flavonols chelate metal ions at the 3-hydroxy-4-keto group (when the A ring is hydroxylated at position 5). An O-quinol group at the B ring can also demonstrate metal chelating activity (Pratt & Hudson 1990). The 3' and 4' groups in the B-ring have only weak Cu-chelating activity (Letan 1966). One or two copper atoms can be bound by chelation to one flavonoid molecule and may be withdrawn from the biological medium in this way. This effect will therefore potentially have an effect on copper dependent or copper containing enzymes.

There is inactivation of ascorbic acid oxidase, a copper enzyme, by flavonoids (Kühnau 1976).

Flavonoids are also good Fe chelators (Rhodes 1996). The iron chelating activity of rutin in lipid peroxidation may be explained by the formation of inactive iron-rutin complexes (ferrous $\rightarrow$ ferric ions into the complex) (Kozlov et al 1994).

1.3.3 Antioxidant activity and free radical scavenging of flavonoids

Fruit and vegetables are protected to some degree against oxidative condition by their natural flavonoids and at least in this sense certain flavonoids do possess an useful nutritive role (Kühnau 1976). They are very strong antioxidants, and may be more potent than other known antioxidants (e.g. Vitamin C and E) (Robak et al 1988a; Salah et al 1995; Chen et al 1996). Flavonoids can inhibit xanthine oxidase which is responsible for superoxide production (Robak and Gryglewski 1988a).

$Fl(OH)+R^{\cdot} \rightarrow Fl(O^{\cdot})+RH$ $\qquad$ $Fl(OH)+O_2^{\cdot -} \rightarrow Fl(O^{\cdot})+HOO^{-}$

Husain et al (1987) show many flavonoids scavenge hydroxyl radicals produced by the photolysis of hydrogen peroxide or in a Fenton system. Hanasaki et al 1994 observed rutin to be 100-fold superior as a hydroxyl scavenger to mannitol. Free radical chelating properties of flavonoids are responsible for inhibition of lipid peroxidation (Korkina & Afanas'ev 1997).

Flavonoids and polyphenols are good scavengers of free radicals due to their hydroxyl substitutes in the hydrogen atom.

Mangiapane et al (1992) suggested catechin, a natural flavonoid inhibits the oxidation of LDL. Rutin inhibited lipid peroxidation and free radical production of neutrophils and macrophages in iron-overloading rats (Afanas'ev et al 1989). Quercetin and other flavonoids also inhibit the in vitro oxidation and cytotoxicity of LDL (De Whalley et al 1990 and Negre et al 1992).

Quercetin, kaempferol, catechin and taxifolin reduced the cytotoxicity of superoxide ion and hydrogen peroxide (Nakayma et al 1993) and morin can protect cells from the human circulatory system against oxygen radical-mediated damage (Wu et al 1995).

The antioxidant property of flavonoids is believed to be due to the presence of phenolic hydroxyl groups on the A and B rings. A very recent publication supports the view that the reactivities of flavonoids increase with increasing number of hydroxyl substitutes in ring B (Korkina & Afanas'ev 1997). All flavonoids with 3', 4'-dihydroxy configuration possess antioxidant activity. Other important features include a carbonyl group at position 4 and a free hydroxy group at position 3 and/or 5 (Dziedzic & Hudson 1983). But the single hydroxyl substitution at position 5 provides no activity (Cao et al 1997). The structure relating antioxidant activities of flavonoids are explained in Chapters 3

and 4.The chemical structures of flavonoids used in this thesis are shown in (**Figure 2.4**) and a summary of antioxidant activities of flavonoids and polyphenols is shown in (**Table 4.**).

1.3.4 Other biological effects of flavonoids

Flavonoids are not a homogenous group of compounds with similar chemical properties. Autoxidation of flavonoids can occur in some situations e.g. in the presence of iron ions or Fe-EDTA (Laughton et al 1989) may be responsible for a cytotoxic activity of these compounds (Korkina & Afans'ev 1997). Mutagenic activity of quercetin in some in vitro (but not in vivo) reports may be induced by metal ions or through degradation of quercetin by superoxide and finally autoxidation. An antimutagenic activity of some flavonoids is known, e.g. rutin inhibited the mutagenic effects on mineral fibres and dusts on human lymphocytes (Korkina et al 1992). This action is believed to be related to antioxidant and chelatory properties.

A wide range of other actions is suggested from in vitro experiments. Flavonoids inhibit platelet aggregation (Herbert 1988) and they have anti-viral and anti-bacterial properties (Rhodes 1996). Quercetin and quercitrin are the most potent inhibitors of aldose reductase known so far, which suggests that they may be useful in preventing the onset of diabetic or galactosemic cataracts, and may potentially oppose diabetic neuropathy and angiopathy. Some of the other biological effect of dietary flavonoids are shown in (**Table 1.2**).

1.4 Food sources of flavonoids

Information about the flavonoid contents of foods comes from a large number of ad hoc reports over many years. A variety of assays have been used, often measuring only the aglycone base. There is much less information about food contents of specific conjugates.

The total daily flavonoid consumption in normal western diets has been estimated at about 1g/day (Kuchnau 1976), or at about 25.9 mg/day from HPLC assays on foods in the Dutch diet. These amounts compare with intakes in the general population of 8-12 mg/day Vitamin E, or 73-74.6 mg/day of vitamin C in the UK (Gregory et al).

1.4.1 Onions

Leighton found quercetin agylcone and quercetin 4'-D-glucoside in onions (Leighton et al 1992). Quercetin compounds from onions have a higher bioavailability than those from tea (Hollman et al 1996a). Red onions contain very high amount of flavonoids (>1000 mg/kg) more than yellow onions (60 mg/kg) and non detectable flavonoids in white onions (Leighton et al 1992). The extracts of red onion skins (0.3%) reduce the peroxide value and act as a commercial antioxidant (Akaranta & Odozi 1986). White skins onions (allium cepa) have less flavonol content than coloured skins. Herrman (1976) has shown outer dry skin of coloured onions contain 2.5 to 6.5% quercetin aglycone.

Contrary to the results of Herrman (1988),Crozier and co-workers (1997a) recently found the red skinned onion did not contain higher levels of quercetin than the white skinned varieties. Also in contrast to the findings of Bilyk et al (1984) they did not detect kaempferol in any of the onion extracts, but they found quercetin (185-634 $\mu g/g^{-1}$ fresh weight onions) very similar to the result of Hertog et al (1992) (**Table 1.3**).

Onions are a major source of dietary flavonoids in some populations, particular when consumption of other sources of flavonoids (e.g. wine and tea) is low. Onions were considered the most important sources of flavonoids at 29% of total after tea in the Netherlands (Hertog et al 1993b), while in Finland onions provide 64% of all dietary flavonoids (Knekt et al 1996). Flavonoids glucosides of onions are better absorbed than the aglycones by human gut in ileostomy subjects (Hollman et al 1995), and this work disproved the earlier belief that flavonoids in foods cannot be absorbed from the intestine because they are bound to sugars as glycosides, and only free flavonoids are able to pass through the gut wall (Kühnau et al 1976). Consumption of onions is related to reduced risk of stomach carcinoma, but not protective against lung carcinoma (Dorant et al 1994).

Quercetin levels in onions show variations between seasons in the range 28.4-48.6 mg/100g in the Netherlands (Hertog et al 1996), and 185 – 634 μg quercetin g^{-1} fresh weight in a Glasgow study (Crozier et al 1997). Rhoudes et al 1996 reported up to 1.2-1.6 mg quercetin glycosides/g fresh weight. Leighton et al

(1992) found that shallots contains very high amount of (quercetin 4'-glucoside, quercetin aglycone, 3-quercetin diglucosides, isorhamnetin and kaempferol monoglycoside) (**Table 1.3**). Using an older assay, the highest quercetin content of 8 varieties of onions was 60 mg/kg, kaempferol 7 mg/kg and no myricetin was detected in a sweet spanish hybrid (Bilyk et al 1984). Two glycosides form of quercetin (quercetin-4'-glucoside and quercetin-3,4'-diglucoside) were purified from onions (Williamson et al 1996).

You et al (1989) by using interviews in 564 patients with stomach cancer in China found the protective effects of onions were seen against stomach cancer.

1.4.2 Tea

Tea is a widely consumed beverage throughout the world and has a wide range of consumption up to 20 cups or more/day. Tea leaves contain more than 35% of their dry weight in polyphenol compounds (Balentine 1992, quoted by Serafini et al 1996). Green tea is a non fermented type of tea which is consumed in China and Japan and black tea is a fermented tea and mostly consumed in the Western world, Asian countries (South and South east) and over the whole of African (Serafini et al 1996). In China, tea has been consumed as beverages and a crude medicine for 4000 years (Ho et al 1992). Tea contains mild stimulants (caffeine, theobromine) and is not usually drunk by children in western cultures, but it is a common weaning food in East Africa.

Tea was the most plentiful source of dietary flavonoids in the Netherlands, kaempferol and quercetin being the main flavonoids (Hertog et al 1993b). Estimates from dutch food analyses suggests that tea provided 82% of total flavonol intake of 1900 Welsh men in the Caerphilly Study (Hertog et al 1997). Black tea contributed about 70% of total flavonoid intake of the Zutphen Study in the Netherlands (Keli et al 1996).

Both black (fermented) and green teas are rich in polyphenols which can increase antioxidant activities in vivo. Adding milk to black tea was suggested to reduce flavonoids bioavailibility (Serafini et al 1996; but unpublished data from Katan do not show any such effect (personal communication). Flavonoid content of tea bags is generally higher than tea prepared with loose leaves, perhaps because the leaf fragments are smaller. Black tea infusions contain quercetin (10-25 mg/L), kaempferol (7-17 mg/L) and myricetin (2-5 mg/L) (Hertog et al 1993b). Quercetin has a higher level in infusions from black tea bags (5 varieties) than kaempferol and myricetin (Hertog et al 1996) (**Table 1.3**). Finger and Englehant (1991) found plenty of flavonol glycosides, quercetin rhamnodiglucoside (0-0.95 g/ kg^{-1}) and kaempferol rhamnodiglucoside (0.5-1.25 g/kg^{-1}) by GC-MS methods in black tea.

Catechin and catechins esters (ECG, EGC and EGCG) are polyphenolic flavonols which act as scavengers of free radical and antioxidants (Salah et al

1995). Quercetin-3-O-β-rutinoside (rutin) is the major quercetin compound in tea, and 17% absorption has been shown in human gut) (Hollman et al 1995). Ho and Co workers isolated four catechins (EC, EGC, ECG and EGCG) from 12 types of green, black and semi-fermented tea. Also they found EGC, EGCG and ECG at the concentration of 10-20 μmol/l antioxidants in the soybean lipoxygenase assay (Ho et al 1992). EGCG in green tea may be cancer protective in humans (Fujiki et al 1996).

Polyphenol theaflavins are responsible for the reddish colour of tea (Serafini et al 1996) and are formed during the manufacture of black tea from the enzymic oxidation of the flavonols, catechin and gallocatechins by polyphenol oxidase. All of these polyphenols are antioxidant (Miller et al 1996; Salah et al 1995). EGCG is a major polyphenol in green tea and is effective in inhibiting DNA single-strand breaks in vitro with different mutagenic substances (Hayatsu et al 1992). Antiomutagenicity and antigenotoxic activities were attributed to green tea polyphenols by Wang et al 1989. Protection against rat liver oxidative DNA damage by epigallo catechin gallate in green tea was shown by Hasegava et al 1995.

1.4.3 Other important source of flavonoids

Flavonoid content analysis of 28 vegetables and 9 fruits commonly consumed in the Netherlands found leafy vegetables to have highest flavonoid levels in summer. Quercetin levels were present in the edible parts of most vegetables were lower than onions e.g. kale, broccoli, french beans and slicing beans. Kaempferol was only detected in kale, endive, leek and turnip (Hertog et al 1992) (**Table 1.3**). Crozier et al (1997a) found large amounts of quercetin in onions, lettuce, cherry tomatoes, Scottish or Spanish or Dutch beef tomatoes, and luteolin and apigenin in celery. They found cooking lowered the quercetin content of both tomatoes and onions with more reductions being detected following microwaving (65% in tomato, 64% in onoins) and boiling (81.7% in tomatoes, 74.6% in onions) then after frying (35.2% in tomatoes, 21.3% in onions) (Crozier et al 1997a). Some natural sources of plant antioxidants contain flavonoids and polyphenols presented in **(Tables 1.1 and Tables 1.3)**. Virtually all fruit and vegetables contains some flavonoids (Bilyk & Sapers, 1985 & 1986), but isoflavones are largely limited to one plant family, the leguminosae (pea family) (Rhodes et al 1996; Shahidi et al 1992). Naringin is the predominant flavanone in grapefruit (citrus paradisi) and hesperidin is the primary flavanone in orange (citrus sinensis), and both are absorbed by the human gut (Ameer et al 1996).

For the present thesis, onions and tea were selected as the principal foods for study, because Hertog et al (1992 and 1993b) had identified these as the most

important contributors to total flavonoid intake in European diets, and because their contents of other known antioxidants (e.g. Vitamin C, E, A, carotenoids) is low. A quantitative analysis of the flavonoid content of commercial fruit and vegetable in Glasgow found high amount of flavonoids, mostly quercetin in onions and tea. Tomato ketchup was used for half the subjects in the intervention study. Tomatoes contain mainly quercetin (Crozier et al, 1997a).

1.5 Relationship between dietary flavonoids and health

Recognising potential links between flavonoids mainly through their antioxidant actions, and health, several analyses have been applied to epidemiological studies. The usual approach has been to apply figures for total flavonoid contents of foods to studies with dietary inventories or food frequency questionnaires. The main problem with this approach is that foods, or dietary patterns, high in flavonoids are likely to be high in other compounds with potential biological activity. Thus dietary flavonoids could be non-causal markers of other dietary or nutrient effects.

1.5.1 Flavonoids and cardiovascular disease

After 5 years of follow up in the Zutphen Elderly Study (Netherlands) there was inverse relation between mortality from CHD and high flavonoids intake (flavonol and flavone >29.9 mg/day) estimated from diet questionnaire and local

flavonoid analysis of key foods (Hertog et al 1993a). The same relation appeared in the the 25 years of follow-up of The Seven Countries Study (Hertog et al 1995). Tea was the major source of flavonols in both studies (Hertog et al 1996), from which it might appear that tea has a special protective role. However, Brown et al (1993) in Scottish people and Grobbee et al (1990) in US people found no relation between CHD and drinking of tea. It is not clear whether positive associations can be caused by flavonoids or other substances in tea e.g. caffeine or alkaloids. It can only be concluded that more studies on the relation of tea, its flavonoids and CHD are necessary.

Recently in the Caerphilly study, Hertog et al (1997) re analysed food frequency data, using Dutch figures for flavonoid contents of foods. They reported the incidence of heart disease mortality over 14 years in 1900 men (in Caerphilly, Wales, UK) went up with tea but down with onion consumption (Katan, Hertog et al 1997), but they could not find any association with their estimate of flavonoid consumption.

Again based on diet records, 20 years follow up in Finland suggested that people with very low intakes of flavonoids have higher risks of coronary disease (Knekt et al 1996). The low incidence of heart disease in France has been related to high flavonoid intake particularly from from red wine (Katan 1997).

The antioxidant properties of flavonols and catechins suggested that their intake might prevent atherosclerosis although other components, or associated patterns of nutrient intake could be reponsible.

Ishikawa et al (1997) showed tea flavonoids added to in vitro protected LDL from copper-induced oxidation and macrophage-mediated oxidation. Also tea consumption decreased oxidative modification of LDL, and may have favourable effects to protect atherosclerosis (Steinberg et al 1989). In the Zutphen Study there were a strong inverse relation between intake of flavones and flavonols and stroke risk (Keli et al 1996). The highest category of flavonol and flavone intake (>30 mg/day) had about one-third the risk of stroke compared to men in the lowest category (Hertog et al 1996).

In conclusion, more research including randomised controlled clinical trials needs to be the solution of questions about relations between CHD and flavonoids. A benefit to cardiovascular health is suggested by some epidemiological data but remains unproven in the absence of experimental studies of atherosclerosis or appropriate clinical trials (Muldoon & Kritchevsky 1996).

1.5.2 Flavonoids and cancer

Quercetin can inhibit growth in vitro of cells from various human cancers such as colon (Ranelli et al 1992), ovarian (Scambia et al 1991) and stomach

(Yoshida et al 1990). Wei and Co Worker (1990) by using ODC assay (ornithine decarboxylase activity) found 20 μmol/l of apigenin inhibited the promotion of papillomas in mice induced by dimethylbenzanthracene (DMBA). Also they reported the inhibition by apigenin (a plant flavonoid) of mutagenesis in the salmonella system (Ames assay). At very high doses, the two most common flavonoids, quercetin and kaempferol, exhibit some mutagenicity in the Ames assay. Probably autoxidation of quercetin and kaempferol interfere during the Ames assay induces a false result. By using ornithine decarboxylase activity assay Kato et al (1983) & Verma et al (1988) observed quercetin (10-30 μmol/l) or apigenin (Wei et al 1990) inhibit tumour promotion. Dietary quercetin at a level of 2% inhibited colon tumour incidence and both rutin and quercetin suppress multiplication of colonic neoplasia induced by azoxymethanol in mice (Deschner et al 1991).

The results of a middle aged cohort study in the Netherlands (n=120,852; age 55-69, during 4.3 years) suggested that intake of quercetin alone or quercetin and other flavonols and flavones was not associated (either postively or inversely) with cancers of the stomach, colon and lung (Hertog et al 1996). According to the results of the 25 years of follow-up in the seven countries study, flavonol and flavone intake (e.g. tea and red wine) at baseline was not related to differences in lung, colorectal and stomach cancer mortality rates (Hertog et al 1995). In other studies, there was not any association between risk of cancer and consumption of red wine (IARC 1988) or consumption of tea (Yang & Wang 1993). Dorant

found an inverse association between cancer of stomach, colon and rectum and consumption of allium vegetables (Dorant et al 1994a and 1996; Hertog et al 1996), but not female breast carcinoma (Dorant et al 1995). However, Kohlmeier et al (1997) did not find relation between tea consumption and risk of cancer. Yoshida et al (1997) suggested that in vitro anti-proliferative effects of quercetin were due to the specific arrest of the G1 phase of the cell cycle.

The exact mechanism responsible for the reported antitumour effect of flavonoids is not yet understood, but it is possible the flavonoids (mainly quercetin) inhibit the growth of malignant cells e.g. with activation of glycolytic enzymes or protein synthesis (Korkina & Afanas'ev, 1997).

In conclusion, several flavonoids have fairly conistent anti-tumour effects in vitro, and at high doses in animal models. Earlier reports of mutagenic effects were probably artefactual. The human evidence, however, provides little support for anti cancer effects. More conclusive research will require better knowledge of dietary flavonoid content and absorption, and of their fats in the body.

1.5.3 Other diseases and flavonoids

In 1936 Györgi separated two flavonoids from citrus fruits and found they can decrease capillary fragility and permeability (thus called vitamin P) in humans (Rusznyak & Szent-Györgyi 1936). The term "Vitamin P" was coined from this study. The explanation may lie in substitution of vitamin C in its antioxidant

role. Flavonoids can inhibit the cyclooygenase activity and thus decrease platelet aggregation and tendency to thrombosis (Laughton et al 1991). Flavonoids demonstrated a wide range of biochemical and pharmacological effects, including anti-inflammatory and anti-allergic effects (Middleton et al 1992). There is not enough information to draw conclusions about any roles for flavonoids in other diseases, but most of the evidence points towards beneficial actions.

1.6 Aims and Research Questions of the thesis

Aims

1. The first aim was to develop and evaluate the SCGE or comet assay as a reproducible method for measurement of the oxidative DNA damage to human lymphocytes (ex vivo), which might be applied in a dietary study.

2. The second aim was to assess the antioxidant activities of food derived flavonoids by observing the protection against DNA damage caused by oxygen-radical. This was a novel application and was used to compare the actions of flavonoids with Vitamin C.

3. The third aim was to establish if dietary flavonoids are absorbed, and if there is any associated improvement in antioxidant defences in diabetic patients.

Several specific Research Questions were addressed:

Research Question 1

Do flavonoids add to the antioxidant activity of vitamin C in the SCGE assay when given in combination?

Research Question 2

What is the relation between the number of hydroxyl groups of flavonoids and their antioxidant activity in the SCGE assay?

Research Question 3

Is there any association between the free and conjugated flavonoids and their antioxidant activity in the SCGE assay?

Research Question 4

How do the SCGE and TEAC assays compare in ranking antioxidant effects of flavonoids?

Research Question 5

What is the flavonoid consumption from normal Scottish diets, and is the range of consumption likely to influence antioxidant status?

Table 1.1 - Major dietary sources of flavonoids and polyphenols

Flavonoids	Major Dietary sources	References
Catechins	Tea, apples, apricots, cherries	Hollman (1997)
Flavanones	Citrus fruits, (grapefruit orange)	Ameer et al (1996) Kühnau (1976)
Flavones	Parsley, thyme	Hertog et al (1992)
Flavonols	Onions, kale, broccoli, apple, cherries, tea , berries	Hertog et al (1993b)
Anthocyanidins	Grapes, cherries	Kühnau (1976)
Isoflavones	Legumes, soybean	Adlercrentz (1993)

Table 1.2 - Some of the biological effects of dietary flavonoids

Effect	Type of flavonoid	Methods/cell	References
Heavy metal chelator	flavonol	copper	Kühnau (1976)
Antibacterial/antiviral (1972)	flavonol	staphylococcus aureus shigella sonnei	Ramaswamy et al
Antitumour	flavonols-flavones	human carcinoma of nasopharynx	Suolinna et al (1975)
Antiproliferative effect	quercetin	human leukemic T-cells	Yoshida et al (1992)
Ascorbic acid synergist	rutin	guinea pig	Kühnau (1976)
Inhibitors of enzymes:			
(a) catechol-O-methytransferase	quercetin	rat mitochondria	Kühnau (1976)
(b) membrane Na^+/ K^+ ATPase	flavonol aglycones	beef heart	Carpenedo et al (1969)
(c) mitochondrial ATPase	flavonol aglycones	mitochondria	Lang et al (1974)
(d) GSH S-transferase activity (cytosolic)	quercetin, kaempferol, morin, luteolin	rat liver	Zhang & Das (1994)
Antimutagen	flavonoids	against mutagenicity of indolic carcinogen formed during cooking of meat	Sanejima et al (1995)

Table 1.3 – Flavonoids content of some vegetables and fruit

Fruit and vegetables	**Concentration of flavonoids**	**Kinds of flavonoids**	**References**
Sweet spanish hybrid (8 varieties) onions	60.7 mg/kg 7 mg/kg	Quercetin Kaempferol	Bilyk et al (1984)
Shallots	>800 mg/kg	(quercetin 4'glucoside), quercetin aglycone (3 quercetin diglucosides), isorhamnetin (kaempferol monoglycoside)	Leighton et al (1992)
Red onions Yellow onions White onions	>1000 mg/kg 60 mg/kg non detectable	Quercetin aglycone	Leighton et al (1992)
Coloured onions	2.5-6.5 y	Quercetin aglycone	Herrman (1976)
White onion	185-634 mg/kg fresh weight	Quercetin	
Red Onion	201	Quercetin	
tomatoes (Spanish cherry)	17-203 mg/kg	Quercetin	
Round lettuce	450 –911mg/kg	Quercetin	
Tomato (Dutch, Scottish, Spanish)	2.2 – 11.2 mg/kg	Quercetin	Crozier et al (1997a)
Celery	191 mg/kg	Apigenin	
Celery	0-40 mg/kg	Luteolin	
English yellow onions	142 " 0.72 " 6.21 "	Quercetin (glycoside & free) kaempferol (glycoside & free) isorhamnetin (glycoside & free)	
Typhoo tea bag infusion (5 min/250 ml)	7.0 mg/L 3.3 mg/L 0.8 mg/L	Quercetin (glycoside & free) Kaempferol (glycoside & free) Isorhamnetin (glycoside & free)	Crozier et al (unpublished data)
Black tea infusions	10-25 mg/L 7-17 mg/L 2-5 mg/L	Quercetin Kaempferol Myricetin	Hertog et al (1997)

Black tea (5 varieties)	17-25 mg/L 13-17 mg/L 3-5 mg/L	Quercetin Kaempferol Myricetin	Hertog et al (1996)
Black tea	50-1250 mg/kg	Kaempferol	Finger & Englehant (1991)
Soy bean	10-20 µmol/l	EGC, EGCG-ECG	Ho et al (1992)
Endive Leek Turnip Kale Brocolli French beans Slicing beans Onions (variation of 3 seasons)	15-91 mg/kg 11-56 mg/kg 31-64 mg/kg 110 mg/kg 30 mg/kg 32-45 mg/kg 28-30 mg/kg 284-486 mg/kg	Quercetin	Hertog et al (1992)
Grapefruit juice	373.1 mg/L 241.1 mg/L 6.5 mg/L 3.2 mg/L	Naringin (glycoside) Naringenin (aglycone) Hesperidin (glycoside) Hesperitin (aglycone)	Ameer et al (1996)
Orange juice	37.6 mg/L 17.6 mg/L 65.0 mg/L 32.2 mg/L	Narirutin (glycoside) Naringenin (aglycone) Hesperidin (glycoside) Hesperitin (aglycone)	Ameer et al (1996)

Table 1.4 - Generation of free radicals

Name	Chemical Formula	Process
Oxygen radical[a]	$O^{\cdot}$	1-5% of all oxygen used in metabolism
Perhydroxyl radical[b]	$HO_2^{\cdot}$	$O^{\cdot} \xrightarrow{\text{low PH}} HO^{\cdot}_2$ (more reactive than $O^{\cdot}$)
Alkoxyl radical[a]	$RO^{\cdot}$	lipohydroperoxy ($ROOH+Fe^{2+} \longrightarrow Fe^{3+}+OH^{-}+RO^{\cdot}$)
Superoxide[b]	$O_2^{-\cdot}$	$O_2 + e \longrightarrow O^{-\cdot}_2$ (by phagocytic cells)
Hydrogen peroxide[b]	H_2O_2**	$O_2+2e+2H^{+} \longrightarrow H_2O_2$
Hydrogen peroxide[b]	H_2O_2**	$2O_2^{-\cdot}+2H^{+} \longrightarrow H_2O_2+O_2$
Hydroxy radical[b,g]	$OH^{\cdot}$	$H_2O_2+Fe^{2+} \longrightarrow {}^{\cdot}OH+OH^{-}+Fe^{3+}$
Hydroxy radical[b]	$OH^{\cdot}$	$O_2^{-\cdot}+H_2O_2 \longrightarrow {}^{\cdot}OH+OH^{-}+O_2$
Superoxide[b]	$O_2^{-\cdot}$	$*Fe^{2+}+O_2 \longrightarrow Fe^{3+}+O_2^{-\cdot}$
Superoxide[b]	$O_2^{-\cdot}$	$Cu^{+}+O_2 \longrightarrow Cu^{2+}+O_2^{-\cdot}$
Hydroxyl radical[d]	$OH^{\cdot}$	$H_2O \xrightarrow{\text{gamma rays}} OH^{\cdot}+H$ (in the body)
Nitric oxide radical[e]	$NO^{\cdot}$	L-arginine $\longrightarrow$ $NO^{\cdot}$ (by vascular endothelium)
Hydroxyl radical[f]	$OH^{\cdot}$	$O_2^{-\cdot}+H \rightarrow O^{\cdot}H+OH^{-}+O_2$ (from hypocholorous acid)
Nitrogen dioxide radicals[c]	$NO^{\cdot 2}$	$NO^{\cdot}+O_2 \longrightarrow NO^{\cdot}_2$ (in cigarette smoke, polluted air)
Trichloromethyl[b,c,d]	$CCl_3^{\cdot}$	$CCl_4 \xrightarrow{\text{P-450}} CCl_3^{\cdot}$ ($CCl_3^{\cdot}+O_2 \longrightarrow CCl_3O_2^{\cdot}$) in the liver

a) Neville et al (1996)
b) Cheeseman & Slater et al (1993)
c) Halliwell (1996)
d) Halliwell (1994)
e) Moncada & Higgs (1993)
f) Candeias et al (1993)
g) Hunt & Nixon (1993)
* Ferrous (Fe^{2+}), Ferric (Fe^{3+})
**) Not free radicals

Table 1.5 - Antioxidant defences against free radicals in humans

Mainly Intracellular	Mainly Extracellular	Both (Intra and extracellular)
Ferritin[f]	Tranferrin (plasma iron binding protein)[a,e]	superoxide dismutase[b]
Catalase[e,h]	lactoferrin (iron binding protein)[a]	Glutathione peroxidase[b,h]
	ceruloplasmin (copper transporter)[a]	Ascorbic acid[b,f,i]
	Uric acid (in plasma)[c]	α-tocopherol[g]
	Haemopexin (bind free haeme)[e]	
	Haptoglobin (bind free haeme)[e]	
	Albumin (bind with SH group)[f]	
	Bilirubin[f]	
	Cartenoids[f]	
	Ubiquinol[f]	

a) Halliwell et al (1990)
b) Neville et al (1996)
c) Stocker R & Frei (1991)
e) Halliwell (1994)
f) Frei et al (1992)
h) Lunec (1990)
i) Sinclair

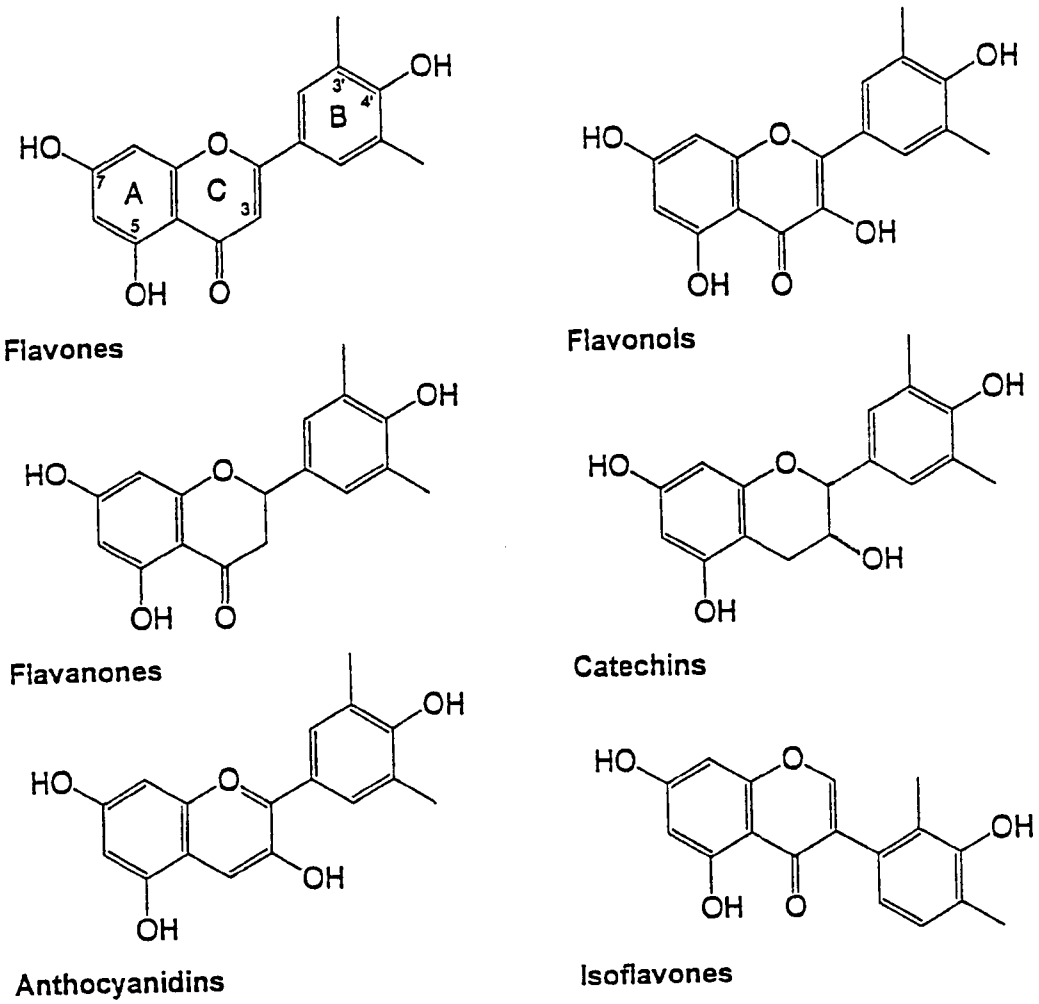


Figure 1.1 Subclasses of flavonoids on based variations in the heterocyclic ring. (Hollman PCH, 1997)

Chapter 2: Methods

2.1 Methods to measure antioxidant activity

Many compounds in the human body have antioxidant activity, which may contribute to biological effects. They can be classified into four main groups:

1. Water soluble antioxidants, including: ascorbic acid, glutathione, uric acid, bilirubin (Frei et al 1992) and D-mannitol (Jeng et al 1994).
2. Lipid soluble antioxidants, including: α-tocopherol, ubiquinol-10, (Kontush et al 1995) lycopene, β-carotene, lutein and oxycarotenoids (Motchinik et al 1994).
3. Protein non-enzyme antioxidants, including: albumin, lactoferrin, ferritin, protein thiols, transferrin, caeruloplasmin, haptoglobin (Langlois 1997), hemopexin (extracellular hemoglobin-binding protein antioxidant mainly β globulin), and N-acetyl cysteine (Satoh & Sakagami 1997).
4. Protein enzyme antioxidants: catalase, Cu & Zn superoxide dismutase, gluthione peroxidase and ceruloplasmin (Frei et al 1992).

It is likely that many other compounds exert antioxidant activity, but these 4 groups make up most of the total antioxidant capacity of plasma (**Table 4.1**). Recent interest in the antioxidant activities of flavonoids, including quercetin and quercetin glucoside (Gugler et al 1975; Hollman et al 1995 and Paganga et al 1997), diosmin and rutin (Paganga et al 1997), catechin (Hollman et al 1997) has added these compounds to the list. Most flavonoids are largely lipid soluble in the free (aglycone) state, but may become water soluble in the conjugated state.

Many assays are described for measuring specific activities of plasma antioxidants and also a variety of methods exist for detection of the total antioxidant capacity of human plasma blood components and cells (Affany et al 1987; Chen et al 1996; Cholbi et al 1991; Galvez et al 1995; Pratt & Hudson 1990; Shahidi et al 1992 and Vinson et al 1995). This variety indicates a lack of an ideal method suited to all compounds or all conditions.

2.1.1 Isolated Compounds

The Fenton reagent assay (Fe^{2+} /H_2O_2) has been widely used for detection of antioxidant activity of isolated substances (Shimoi et al 1994), based on a purely in vitro chemical reaction. In the present thesis, in vitro Trolox equivalent antioxidant capacity (TEAC) assay was used to rank the potency of 17 flavonoids chosen either because they are common in foods or to provide a range of structures, and the standard technique was adapted to examine the influence of flavonoids on the antioxidant capacity of human plasma.

This may not relate directly to antioxidant activity against free-radical-mediated damage in vivo. The lipoprotein oxidation model (Chen et al 1996, Galvez et al 1995, Vinson et al 1995, Shahidi et al 1992 and Pratt & Hudson 1990) has greater biological validity but requires sophisticated GLC measurement.

Measurements of DNA damage are good markers of oxidative damage in isolated cell nuclei. Detection of DNA damage by different methods has

recently been presented as a new and interesting area (Halliwell et al 1997). A number of the toxic processes, e.g. cytotoxic drugs, can cause DNA damage. Oxidative damage to DNA occurs firstly through oxidation of pyrimidine bases, and then by production of DNA strand breaks.

Single cell gel electrophoresis (the comet assay) is a sensitive and rapid method to detect DNA breaks at the individual cell level (Mackelvey-Martin et al 1993 and Fairbairn et al 1995) and specifically for detecting oxidative DNA strand breaks (Collins et al 1995 and Singh et al 1988). It is considered a useful tool for investigating issues related to a standardised oxidative stress in human lymphocytes and to quantify the protection from possible antioxidants (Green et al 1992) but has not previously been used with flavonoids. A range of cell types can be employed, e.g. hepatocytes, sperm, cultured cell line, but the present thesis used normal human lymphocytes in the comet assay to evaluate the antioxidant capacity of some major dietary flavonoids, with vitamin C as a positive control. The standardised oxidative stresses most commonly used are from gamma rays, X rays, UV light and H_2O_2.

In parallel with the standard comet assay, we treated extra slides of patients lymphocytes with endonuclease III, in order to estimate the existing oxidative damage acquired over previous days. This enzyme introduces breaks in the DNA at sites of oxidised pyrimidines and breaks are detected by adaptation of

the comet assay (single cell gel electrophoresis). This test was applied after low and high flavonoid diets in every subject, to look for differences.

2.2 Single cell gel electrophoresis (Comet assay)

2.2.1 Principle

Low concentrations of hydrogen peroxide cause oxidative damage and strand breaks in lymphocyte DNA. Under alkaline conditions, DNA loops containing breaks lose supercoiling, unwind, and are released from the nucleus. They can be visualised as a "comet tail" after gel electrophoresis. DNA strand breaks are thus visualized by the comet assay (**Figure 2.1, 2.2, 2.3**) and can be quantified by image intensification and computer analysis or by visual grading. Pre-treatment of lymphocytes with antioxidant compounds would be expected to decrease H_2O_2 induced DNA damage, and this forms the basis for using the comet assay to assess antioxidant capacity.

2.2.2 Chemicals, solutions and materials

The solutions and chemicals used in this study were purchased from the following companies: High melting point agarose (HMP) and low melting point agarose (LMP) both electrophoresis grade from Gibco Ltd., Paisley, Scotland; Phosphate-buffer saline (PBS) tablets, L-ascorbic acid, kaempferol, quercetin, apigenin, myricetin, rutin (quercetin-3-D-rutinoside), ethylenediamine tetraacetic

acid disodium salt dihydrate (EDTA Na_2), trypan blue solution (0.4%), RPMI (1640) medium with $NaHCO_3$ without L-glutamine and phenol red, Histopaque-1077 (Ficoll), ethidium bromide (EtBr) and diamidine-2-phenylindol-dihydrochloride (DAPI) all from Sigma Chemicals Co. Ltd., Irvine, Scotland; fetal calf serum (FCS) from Globepharm Ltd., Esher, Surrey, England; sodium hydroxide, hydrogen peroxide, Triton X-100, sodium chloride from BDH Chemicals Co. Ltd., England; Tris from Boehringer, Mannheim Ltd., Sussex, England; quercetin-3-glucoside, quercitrin (quercetin-3-L-rhamnoside) and luteolin provided by Apin Chemicals Co., Ltd., Oxon, England. Fully frosted Dakin microscope slides were supplied by Richardson Supply, London, England.

2.2.3 Procedure

Fasting blood samples were drawn from the antecubital vein of healthy volunteer. Fresh peripheral human lymphocytes were isolated by centrifugation with Histopaque 1077 (Ficoll) and incubated with different concentrations (0, 7.6, 23.2, 93 and 279.4 μmol/l) of flavonoids or vitamin C for 30 minutes at 37°C. After pretreatment, cells were washed with phosphate-buffered saline and were treated with H_2O_2 (100 μmol/l, 5 min on ice). Then cells were suspended in low-melting-point agarose set on a frosted Dakin microscope slide and lysed with lysis solution containing 1% Triton X-100 for 1 h. Gel electrophoresis was then used to estimate tail DNA content of 600-1200 lysed nuclei (comets) at each concentration. Visual scoring was used routinely, validated using an Imaging Research BRS2 Image Analyser (Imaging Research Inc., Ontario,

St Catherine's, Canada), with the fluorescence dye ethidium bromide to quantify comet tail DNA.

2.2.4 Cell preparation

Human lymphocytes were isolated from fresh whole blood by adding 30 µl blood to 1 ml RPMI 1640 + 10% FCS on ice for 30 minutes, then underlaying with Histopaque 1077 (Ficoll) before spinning at 200 G for 3 minutes at 4°C. Lymphocytes were separated as a pink layer at the top of the Histopaque.

2.2.5 Antioxidant pre-treatment and wash cells

Cells were incubated with different concentrations of flavonoids or vitamin C for 30 minutes at 37°C in a dark incubator together with untreated control samples. Samples were then spun at 200 G for 3 minutes at 4°C. After pre-treatment cells were spun and washed twice with PBS (0.01 M) at 200 G for 3 minutes at 4°C.

2.2.6 Oxygen-radical treatment

Samples were suspended in PBS with 100 µmol/l hydrogen peroxide for 5 minutes on ice in the dark. Samples were then spun at 200 G for 3 minutes at 4°C. Control samples were treated with PBS alone without hydrogen peroxide.

2.2.7 Slide preparation

Two layers of agarose were prepared. For the first layer, 85 µl of 1% high melting point agarose (HMP) or standard agarose prepared at 40°C in PBS was

dispensed onto fully frosted slides and covered with a 22 x 22 mm (No. 1) coverslip. To solidify the agarose, the slides were stored at 4°C for 10 minutes. Lymphocytes were suspended in 1% low melting point agarose (LMP) in PBS (prepared at 37°C) and 85 µl containing approximately 20,000 lymphocytes were plated out on to the first layer of agarose, covered with a coverslip and stored for 10 minutes at 4°C to solidify. After removing the coverslips the slides were immersed in freshly prepared cold lysing solutions.

2.2.8 Cell-lysis

Slides were treated at 4°C for 60 minutes (vertically without cover slip) with lysis solution of 2.5 mol NaCl, 100 mmol/l Na_2EDTA and 10 mmol/l Tris, adjusted to pH 10 with NaOH plus 1% Triton X-100 (added immediately before use). Different lysis tanks were used for control slides. The lysis solution was stored at 4°C before use.

2.2.9 Alkaline treatment

After the slides were removed from the lysis solution, they were placed in a electrophoresis tank horizontally side by side. Up to 18 slides, in two rows of 9, were electrophoresed simultaneously. Any gaps were filled with blank slides to avoid spaces between slides. Slides were covered with fresh electrophoresis buffer (300 mmol/l NaOH and 1 mmol/l Na_2EDTA, pH 13) at 4°C for 40 minutes to a depth of 2-3 mm above the slides. Buffer was made up freshly

each day and stored at 4°C prior to use. To prevent additional DNA damage from light, slides were processed in dark conditions.

2.2.10 Electrophoresis, Neutralising and Staining

The electrophoresis was run at 25V for 30 minutes at 4°C, covered with black paper against light. Before staining, slides were placed vertically without a cover slip in a neutralising tank and gently washed 3 times for five minutes with neutralising buffer (0.4 mmol/l Tris adjusted to pH 7.5 with HCl) at 4°C in the dark. To stain, 60 µl of 20 µg/ml of 4` 6 Diamidine-2-phenylindol dihydrochloride (DAPI) or 60 µl of ethidium bromide (EtBr , 20 µg/ml) was dispensed directly onto slides and covered with a cover slip. Slides were kept for up to 12 hours in a dark and air-tight moist chamber to prevent drying of the gel before viewing.

2.2.11 Quantification of DNA damage

Slides were examined at 400x magnification on an Olympus fluorescence microscope with excitation at 520 nm and a 620 nm emission barrier filter. As the study involved the individual assessment of DNA damage in more than 30,000 cells, it was necessary to develop a rapid visual scoring system. Cells were assigned a score on a five-point scale (range 0-4) according to the amount of DNA in the tail of the comet as estimated by the observer (**Figure 2.1**).

To validate this system, objective measurements of the distribution of DNA were performed for a sample of cells using an Imaging Research BRS2 Image Analyser. These measurements were conducted by quantifying the fluorescent intensity distribution of the comet as a function of distance from the leading edge of the head (**Figure 2.2**).

There was a close relationship between the subjective visual score and the measurements of the percentage of DNA in the tail by image analysis as shown in (**Figure 2.3**). In 90 per cent of cells, the percentage of DNA in the tail for different visual grades of damage fell in the following ranges:

Grade 0 (no damage), < 5%; Grade 1 (low damage), 5-25%; Grade 2 (medium damage), 25-45%; Grade 3 (high damage), 45-70%; Grade 4 (very high damage), > 70%.

2.2.12 Slide scoring

Randomly selected lymphocytes were visually graded for each slide. A total damage score for the slide was derived by multiplying the number of cells assigned to each grade of damage by the numeric value of the grade, and summing over all grades (giving a maximum possible score of 400, corresponding to 100 cells of grade 4). In a given experiment, duplicate slides were prepared and scored for each concentration of the antioxidant. Experiments were repeated three to six times. Therefore, for each concentration of each antioxidant, 6-12 samples of 100 randomly selected cells were analysed in total.

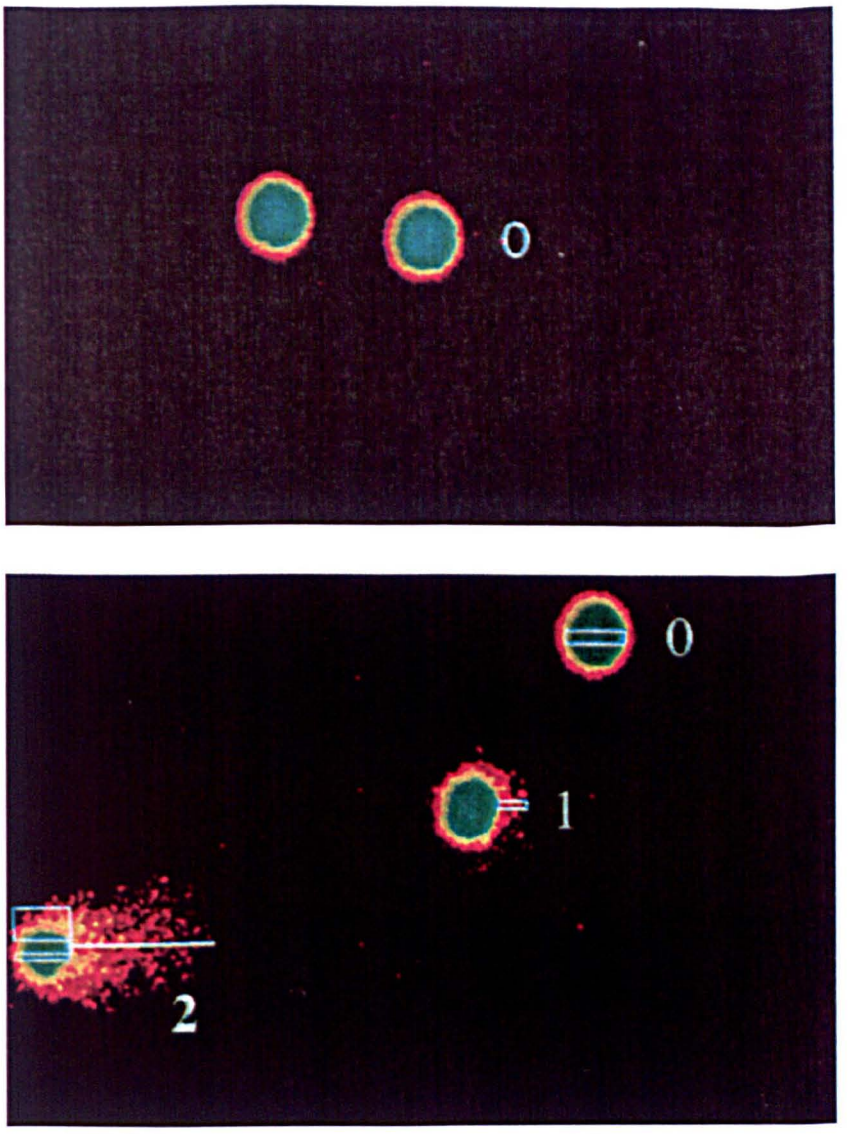
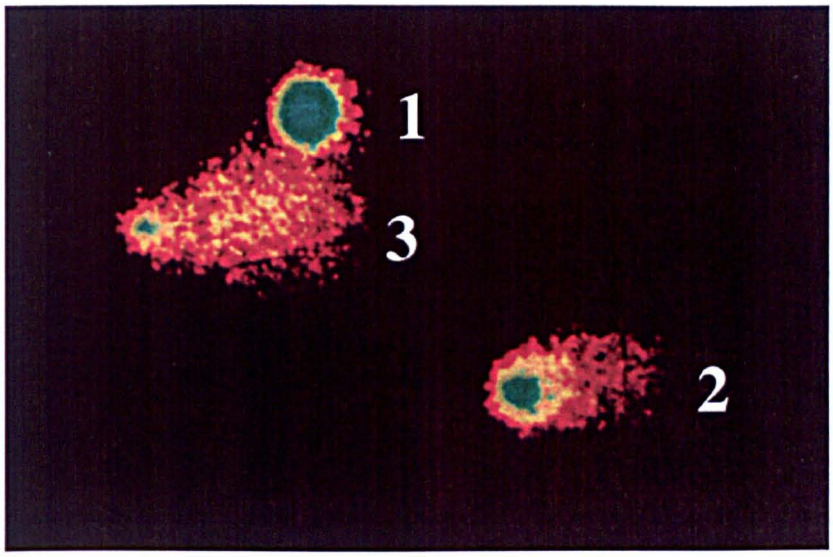
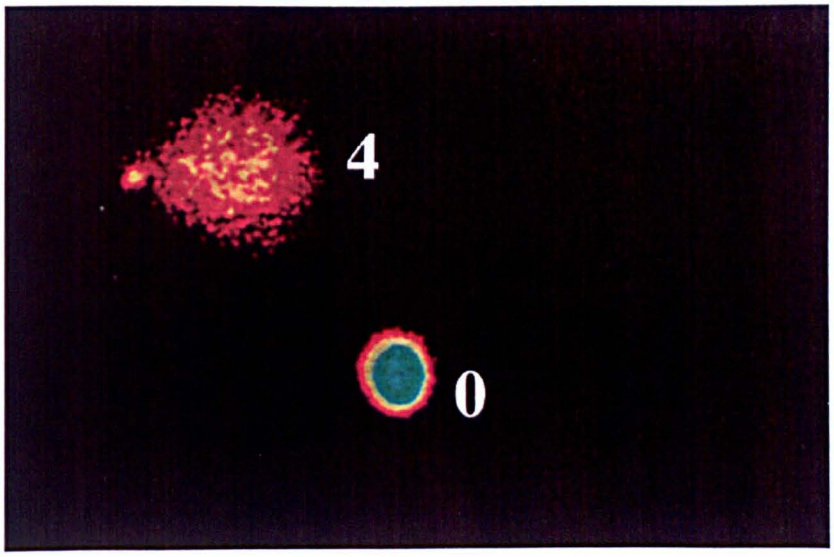


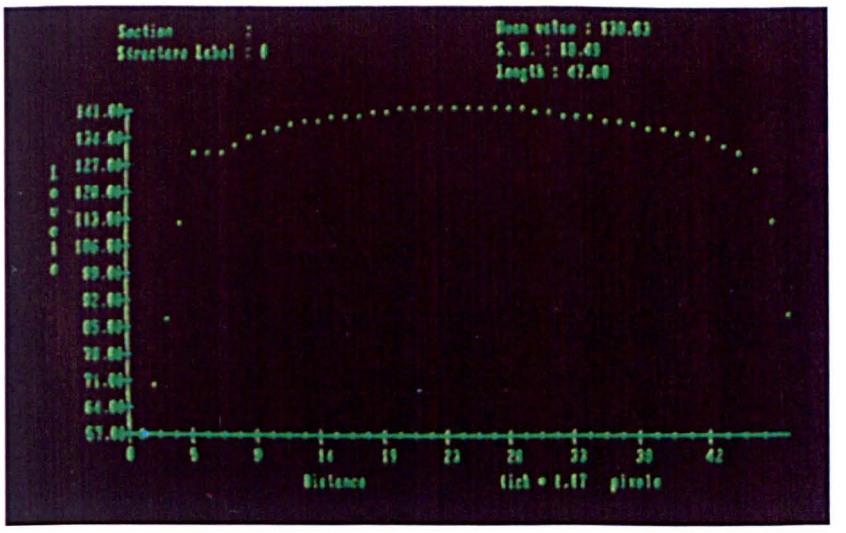
FIGURE 2.1 Human lymphocytes showing varying degrees of DNA damage following treatment with 100 μmol/L hydrogen peroxide. The grade of damage was visually assessed on five-point scale: Grade **0** (no damage), < 5%; Grade **1** (low damage), 5-25%; Grade **2** (medium damage), 25-45%; Grade **3** (high damage), 45-70%; Grade **4** (very high damage), > 70%.


1
3
2


4
0

Grade 0:

(No DNA damage)



Grade 1:

(Low DNA damage)

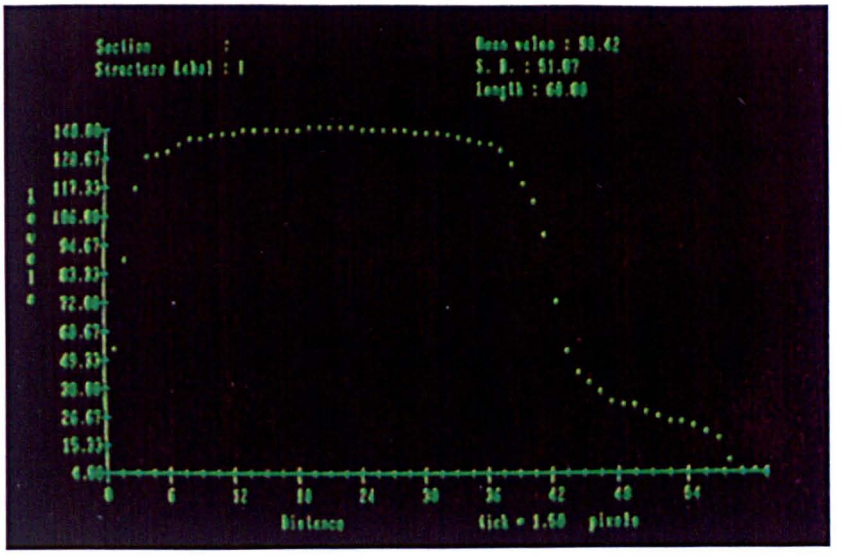
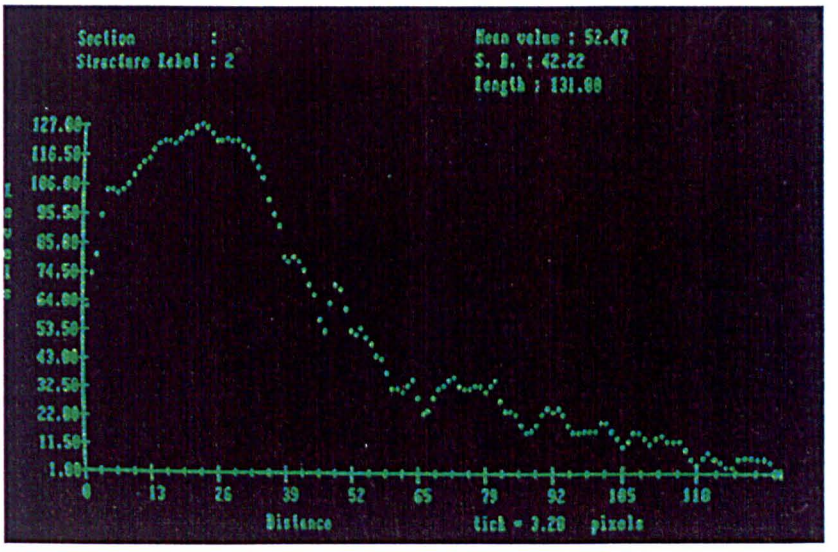


FIGURE 2.2 Fluorescent intensity profiles of comets with different grades of damage as measured by image analysis. Intensity is recorded in arbitrary units. The percentage of DNA in the tail of the comet was calculated as 100 (A_1 - $2A_2$)/A_1, where A_1 = area under curve for whole comet and A_2 = area under curve from leading edge to centre of head of comet.

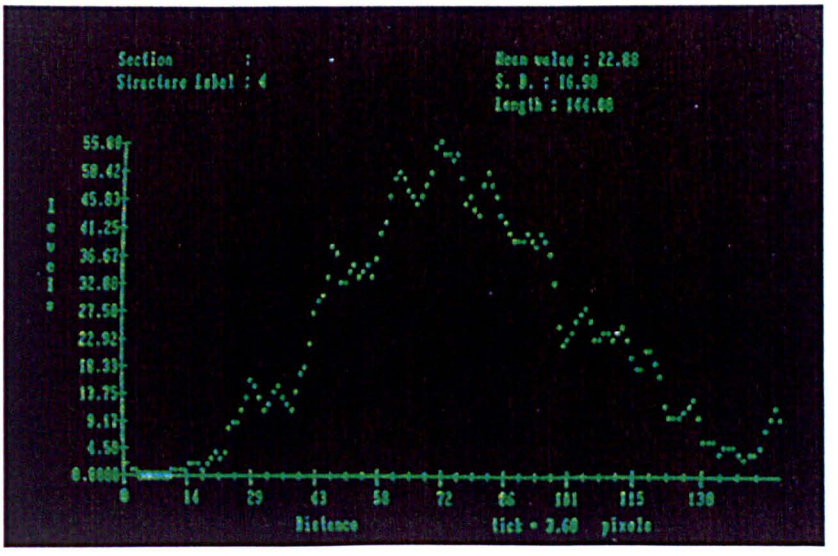
Grade 2:

(Medium DNA damage)



Grade 4:

(Very high DNA damage)



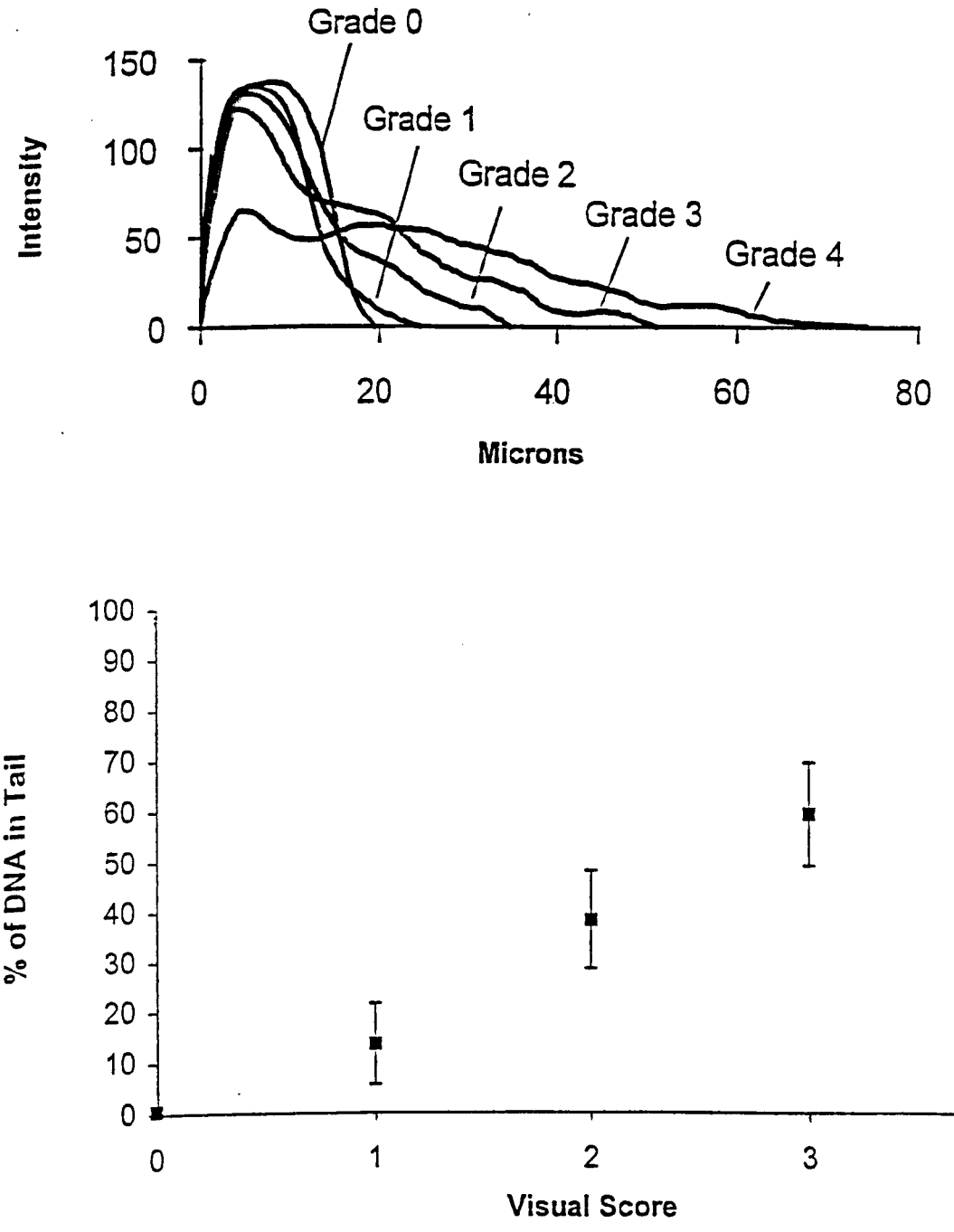


FIGURE 2.3 Relationship between the subjective visual score and the measurements of the percentage of DNA in the tail by image analysis. Each point represents (Mean±SD) for 8-22 comets.

2.3 Endonuclease III assay to detect endogenous oxidative pyrimidine base damage in human lymphocytes

2.3.1 Principles

The bacterial enzyme endonuclease III is specific for oxidised pyrimidines, making a break in the DNA at site of damage. The endonuclease III assay and adaptation of the comet assay is used to detect the presence of oxidative DNA damage received already in vivo in lymphocytes, by converting oxidised bases to strand breaks using the enzyme, which specifically nicks DNA at sites of oxidised pyrimidines (Collins et al 1995). Endonuclease III thus introduces breaks in the DNA at sites of oxidised pyrimidines and breaks are detected by the comet assay (single cell gel electrophoresis).

2.3.2 Chemicals

Endonuclease III is purified by method of Asahara et al (1989) and modified by Collins et al (1993). The solutions and chemicals used in this study were purchased from the following companies:

Purified endonuclease III (EC 3.1.25.1) was received by collaboration with Rowett Research Institute, Aberdeen (Collins et al 1993). Hepes (#H-3375), Bovine Serum Albumin (BSA) (#A-9418) and Na2EDTA (#A-9418) from Sigma Chemicals Co Ltd, Irvine, Scotland. KCL and KOH from BDH Chemicals Co Ltd, England. Other solutions and chemicals were purchased with the comet assay.

2.3.3 Procedures

Fresh peripheral human lymphocytes were isolated from fresh whole blood by adding 30 µl blood to 1 ml RPMI 1640 + 10% FCS on ice for 30 minutes, then underlaying with histopaque 1077 before spinning at 200 g for 3 minutes at $4^{o}C$. Lymphocytes were separated as a pink layer at the top of the histopaque. Slide preparation and cell-lysis were the same procedure as with the comet assay.

2.3.4 Treatment with Endonuclease III enzyme and buffer

Following lysis lymphocytes slides were washed three times for 5 minutes each in endonuclease III buffer (40 mmol/l HEPES - KOH), 0.1 mmol/l KCl; 0.5 mmol/l EDTA; 0.2 mg/ml BSA (bovine serum albumin), adjust PH 8.0, drained and the agarose covered with 50 µl of either endonuclease III in buffer, (1 µg of protein/ml) or buffer only, then slides sealed with a cover slip and incubated for 30 minutes at 37°C (Collins et al 1993). Alkaline treatment, electrophoresis, neutralising, staining, quantification of DNA damage and slide scoring followed as explained before in the comet assay.

It was not possible to check the activity of the endonuclease III independently, but results obtained on healthy normal lymphocytes were similar to those reported by Collins (personal communication).

2.4 Trolox equivalent antioxidant capacity (TEAC assay)

2.4.1 Principles

TEAC assay was used, based on the method of Miller et al (1993). Briefly, metmyoglobin is incubated with H_2O_2. The ferrylmyoglobin produced reacts with the chromogen ABTS (2, 2' - Azino-di- [3-ethyl benzthiazoline sulphonate] to form a radical cation which has a blue colour. Colour production at a fixed time is inversely proportional to the antioxidant capacity of the fluid being studied. The presence of any antioxidant in the reaction mixture delays the production of colour.

Metmyoglobin + H_2O_2 → Ferrylmyoglobin + H_2O

Ferrylmyoglobin + ABTS → $ABTS^{\bullet+}$ (blue-green colour)

2.4.2 Chemicals and Solutions

The solutions and chemicals used in TEAC assay of flavonoids were purchased from the following companies: kaempferol, L-ascorbic acid, quercetin, apigenin, myricetin, rutin (quercetin-3-rutinoside), quercitrin (quercetin-3-L-rhamnoside), luteolin, cathecin, naringenin, pyridine, chrysin, epigallocatechin gallate, hesperidin, epicatechin gallate, silymarin, naringin were purchased all from Sigma Chemicals Co. Ltd., Irvine, Scotland; hydrogen peroxide from BDH Chemicals Co. Ltd., England; quercetin-3-glucoside and isorhamnetin from Apin Chemicals Co. Ltd., Oxon, England.

The flavonoids and polyphenols chosen provided a range in the number of hydroxyl groups from 2 to 8 **(Figure 2.4)**. For measurement of the total antioxidant capacity used a rapid and sensitive TEAC assay (CV=2.1%), available on commercial kit (Randox # 2332, Crumlin, Co antrim, Ireland, UK).

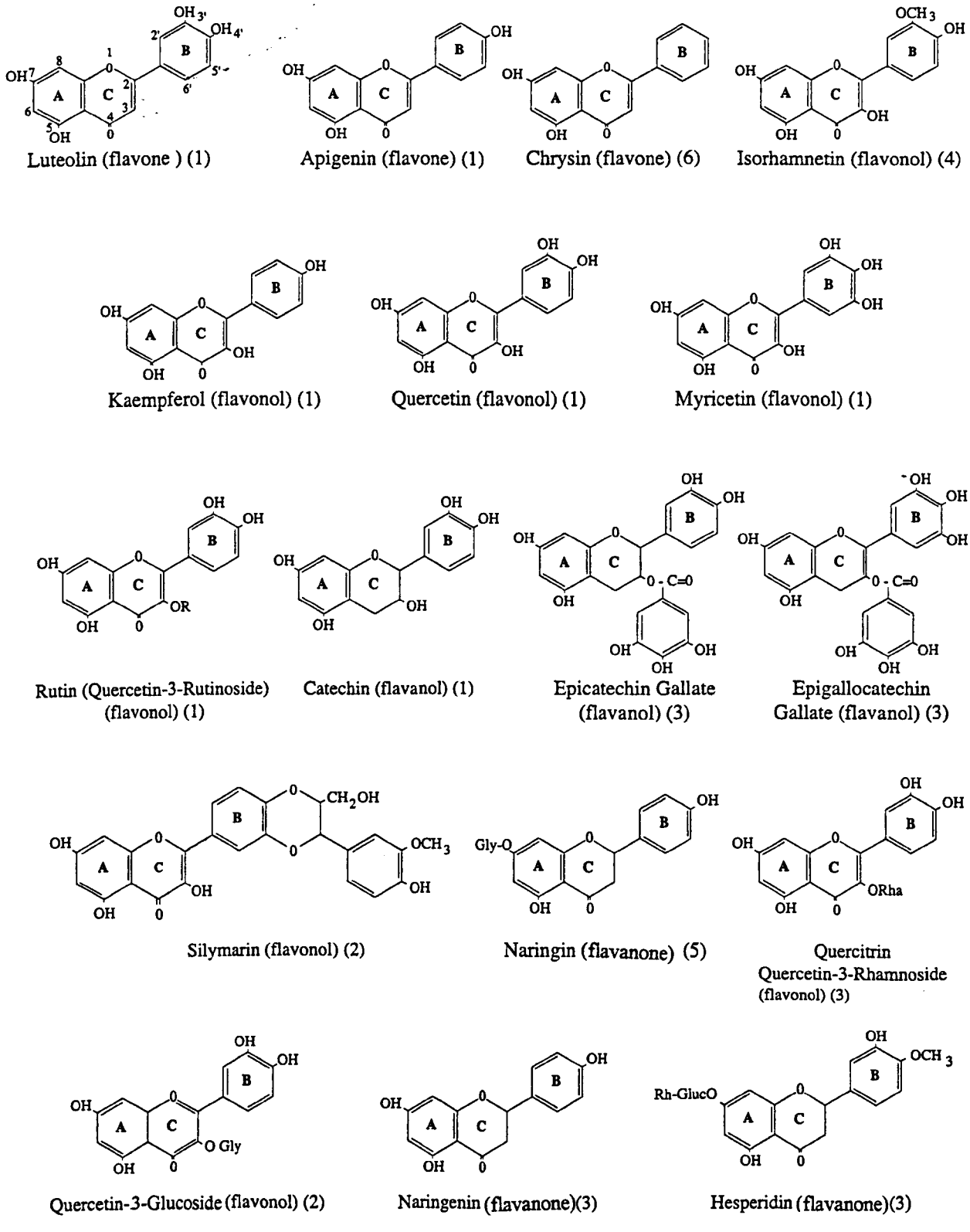


Figure 2.4 The structures of flavonoids and polyphenols used in the present study

(1) Kandaswami C. et al (1994) (3) Shahidi F. et al (1992) (5) Limasset B. et al (1993)
(2) Harborn J. et al (1975) (4) Robak J. et al (1988) (6) Vinson J. (1995)

2.4.3 Procedures

Trolox (6-hydroxy - 2,5,7,8 - Tetramethylchroman, a water soluble vitamin E analogue) is used as a standard. Trolox is twice as potent on a molar basis as vitamin E. Spectrophotometric measurements were made on a Roche Cobas Mira Discrete Analyser. The TEAC of test compounds are expressed as the millimolar concentration of a Trolox solution having the antioxidant capacity equivalent to a 1.0 mmol/l solution of the substances under investigation.

For in vitro measurement of flavonoids all substances were dissolved in absolute ethanol at $37^{o}C$, except hesperidin which was dissolved in pyridine at $\simeq 45^{o}C$. Total antioxidant activity of absolute ethanol was found to be negligible (0.07 ± 0.01 μmol/l).

In the standard assay compounds are tested at a concentration of 1 mmol/l. Since several of the flavonoids in the present study exceeded the maximum TEAC value of 2.5 mmol/l, all samples were tested at the reduced concentration of 300 μmol/l compared with a conventional TEAC at 1 mmol/l, all values were multiplied by 3.33. All solutions were prepared at the same time and measured on the day of preparation. For experiments in human plasma, solutions of quercetin, rutin and kaempferol in ethanol were added to fresh plasma to achieve a final concentration of 10, 20, 50 or 100 μmol/l.

Purity tests of quercetin aglycone, plasma and urine quercetin were measured by reversed-phase high performance liquid chromatography in collaboration with the Institute of Biomedical and Life Sciences, University of Glasgow and is described in Chapter 2, part 2.5 of the thesis.

For TEAC tests on human plasma, fasting heparinised plasma from healthy volunteers (10 male, age 25-37 all non smokers) was freshly prepared by centrifugation of venous blood samples at 3000 rpm for 10 minutes, and measurements on day of preparation. Flavonols were added from stock solutions in ethanol, to a final concentration 10, 20, 50 and 100 μmol/l.

2.5 Determination of flavonoids in plasma, urine and food (test meal)

2.5.1 Introduction

Concentrations of free and conjugated flavonoids in plasma, urine and food (test meal) were determined by reversed-phase (RP) HPLC through collaboration with Miss Jennifer Burns and Dr Alan Crozier at the Institute of Biomedical and Life Sciences, University of Glasgow (Crozier et al 1997b) and methods were as follows:

2.5.2 Extraction and hydrolysis conditions

The test meals, tea, serum and urine samples were hydrolysed using a 3 ml glass V-vial. A teflon coated magnetic stirrer was added to the vial and sealed tightly with a PTFE-faced septum prior to heating at 90^{o}C for the required time.

The pre and post hydrolysed serum and urine samples are centrifuged at 13000 rpm for 10 minutes and 100 µl aliquots of serum and urine samples, taken both before and after hydrolysis were made up to 250 µl with distilled water adjusted to pH 2.5 with Trifluoracetic acid (TFA), prior to the analysis of 200 µl volumes by gradient elution reversed phase HPLC.

In the case of the test meals and tea, extract aliquots of 100 µl, taken before and after hydrolysis, were filtered through a 0.45 µm filter (Whatman, Maidstone, Kent, UK) and made up to 250 µl with distilled water adjusted to pH 2.5 with TFA prior to the analysis of 100 µl volumes by gradient elution RP HPLC. All samples were hydrolysed and analysed in triplicate.

2.5.3 High performance liquid chromatography

Samples are analysed using a Shimadzu (Kyoto, Japan) LC-10A series automated liquid chromatography system comprising of a SCL-10A system controller, two LC-10A pumps, a SIL-10A autoinjector with sample cooler, a CTO-10A column oven, an SPD-10A UV-VIS detector and an RF-10AXL spectrofluorimetric detector linked to a Reeve Analytical (Glasgow, UK) 2700 data system. Reversed phase separations are carried out at 40^{o}C using a 150 x

3.0 mm (internal diameter) C18 separation column (Genesis, Jones Chromatography, Mid-Glamorgan, UK), with a 4 μm C_{18} guard cartridge in an integrated holder.

The mobile phase was a 20 min, 20-40% gradient of acetonitrile in water adjusted to pH 2.5 with trifluoroacetic acid (TFA) pumped at a flow rate of 0.5 ml/min. Column eluent is directed first to the SPD-10A absorbance detector set at 365 nm after which postcolumn derivatisation is achieved by the addition, of 0.1 M aluminium nitrate in methanol containing 7.5% glacial acetic acid pumped at 0.5 ml/min by a pulse-free reagent delivery unit (Reeve Analytical). The mixture is passed through a 0.02" i.d. x 200 cm coil of peek tubing at 40^{o}C before detection of fluorecent flavonoid complexes with the RF-10AXL fluorimeter (excitation 425 nm, emission 480 nm).

The reversed phase HPLC system separates a range of flavonoid conjugates and aglycones, all of which can be detected spectrophometrically at 365 nm. The limit of detection was <1 ng/g food (< 10 ng/ml plasma or urine) and linear 5-250 ng calibration curves can be obtained for morin, quercetin, kaempferol and isorhamnetin. The fluorescence intensities of the individual flavonoid derivatives vary, however, 100 pg-100 ng linear calibration curves can be obtained for morin, quercetin, kaempferol and isorhamnetin.

2.5.4 Hydrolysis techniques

2.5.4.1 Tissue hydrolysis

20 mg powdered, freeze dried tissue

1.6 ml 60% methanol + 20 mM diethyldithiocarbamic acid (antioxidant)

400 µl 6M HCl

20 µl of 500 µg/ml morin Internal Standard

Hydrolysed at 90^{o}C for 1.5 hours with continuous stirring

(result - 1.2M HCl and 50% methanol)

2.5.4.2 Tea Hydrolysis

450 µl liquid tea, made using a standard infusion method

1200 µl 80% methanol + 20 mM diethyldithiocarbamic acid (antioxidant)

300 µl 6M HCl

Hydrolysed at 90^{o}C for 2 hours with continuous stirring

(result – 0.9M HCl and 50% methanol)

2.5.4.3 Plasma hydrolysis

300 µl plasma

500 µl 100% methanol + 20mM diethyldithiocarbamic acid (antioxidant)

200 µl 6M HCl

Hydrolysed at 90^{o}C for 3 hours with continuous stirring

(result - 1.2M HCl and 50% methanol)

2.5.4.4 Urine hydrolysis

0.75 ml urine

1.25 ml 100% methanol + 20 mM diethyldithiocarbamic acid (antioxidant)

0.50 ml 10M HCI

Hydrolysed at 90^{o}C for 2 hours with continuous stirring

(result - 2M HCI and 50% methanol)

2.5.5 Calculations of flavonoids in plasma, tea and test meal

2.5.5.1 Plasma

All the plasma results are expressed in ng of flavonoid per ml of plasma.

2.5.5.2 Tea

The tea results are determined from the the fluorimetric analysis of the sample. They are in flavonoid per μg/ml of tea. Multiplying by 250 will give an estimate of the amount of flavonoids present in 1 mug of tea.

2.5.5.3 Test meals

All the results are given as flavonoid μg/g fresh weight of the test meal. Since each person ate 400 g per day then multiplying by 400 will give the amount of flavonoids ingested per day.

2.6 Human plasma and urine biochemistry including antioxidant measurement

These routine analyses were conducted by staff under the supervision of Dr Dinesh Talwar and Dr Naveed Sattar, Institure of Clinical Biochemistry, University of Glasgow, Glasgow Royal Infirmary. Methods are described only in outline.

2.6.1 Vitamins

2.6.1.1 Simultaneous determination of Vitamin A and E and carotenoids in plasma by reverse phase HPLC

Principle

Heparinized plasma is deproteinised with ethanol containing internal standards. After centrifugation vitamin A and E, carotenoids, and internal standards are extracted with hexane. The hexane is evaporated and the residue dissolved in the developing solvent. An aliquot of this solution is injected on to a C_{18} chromatographic column and vitamin A, E and carotenoids detected at 325 nm, 290 nm and 450 nm respectively. (Talwar et al – in press).

2.6.1.2 Determination of vitamin C in plasma

Principle

Protein is precipitated from heparinized plasma with trichloroacetic acid. The oxidised form of ascorbic acid and dehydroascorbic acid is then coupled with

2,4, dinitrophenyl hydrazine to form the 2,4 dinitrophenylosazone (DNPH). Treatment of the osazone with sulphuric acid causes rearrangement which yields a reddish complex which is measured at 520 nm using a Phillips spectrophotometer (Denson & Bowers 1961).

Table 2.1 Laboratory Reference ranges, obtained in a survey of healthy Glasgow residents (n=111)

Vitamins	**Range (μmol/l)**	**%Coefficient of variation (inter batch)**	**Minimum detectable concentration (μmol/l)**
Vitamin A	1.4 – 2.6	8.1	0.3
Vitamin E	22 – 37.2	9.0	2.5
Vitamin C	11-114	12.6	>10
Lutein	0.15 – 0.37	8.5	0.019
Lycopene	0.19 – 0.55	11.0	0.028
α-carotene	0.03 – 0.11	13.0	0.028
β-carotene	0.18 – 0.58	9.6	0.028
β-cryptoxanthin	0.14 – 0.36	8.7	0.019

Talwar D et al (in press)

2.6.2 Antioxidant enzyme measurements

2.6.2.1 Glutathione Peroxidase in plasma

Principle

GSH-Px activity is determined using the coupled enzyme procedure shown below. The second enzyme is glutathione reductase (GSSG-R). The substrates for GSH-Px are reduced glutathione (GSH) and butyl hydroperoxide. The substrates for (GSSG-R) is oxidase glutathione (GSSH). The reaction is followed by measuring the decrease in absorbance at 340 nm due to the decreasing concentration of NADPH.

$$2\,GSH + ROOH \xrightarrow{GSHPx} ROH + GSSH \xrightarrow{GSSG\text{-}R} 2\,GSH$$

Sensitivity was to 1 unit/g Hb. GSH-Px was adapted on to an automated instrument the spectrophometric method (Cobas Mira, Roche Company) (Beatler et at 1977).

2.6.2.2 Superoxide dismutase activity of plasma

Principle

Superoxide dismutase (SOD) activity was determined using an assay kit from Calbiochem (Nottingham, UK). This kit makes use of a proprietary reagent that undergoes alkaline auto-oxidation which is accelerated by SOD.

Interference from haemoglobin was eliminated by precipitation prior to assay using ice-cold ethanol/chloroform (62.5/37.5 v/v) followed by centrifugation at 3000 g for 5 min at 4^{o}C. SOD activity was determined from the presence/absence ratio of the auto-oxidation rates measured in the presence (Vs) and absence (Vc) of plasma. The data obtained was expressed as SOD activity units per ml of plasma. One SOD activity unit is the activity that doubles the auto-oxidation background (Vs/Vc = 2) sensitivity is 0.2 U/ml and CV = <5% (Nebot et al, 1993).

2.6.3 Selenium in plasma

Principle

The method used was a direct determination of selenium in plasma by electrothermal atomic absorption spectrometry with deuterium-arc background correction. Samples are diluted (1 + 2) with a modifier containing palladium nitrate and Triton X-100. Samples are atomised from a L'vov platform in a pyrolytically-coated electrographite tube and peak area signals are measured. Selenium standards are matched to the physiological concentrations of sodium chloride, calcium and phosphate. The detection limit was 6 μg/L in the original sample, C.V.=5.0% (Gardiner et al 1995).

2.6.4 Human plasma and urine routine biochemistry

2.6.4.1 Microalbuminuria

Principle

The method was an immunoturbidometric test for the quantitative determination of human albumin in urine. A 24 hour urine collection was made in a plain bottle. Samples were analysed within 24 hours. The reagent was obtained from Behringwerke AG, Marbury, Germany and anti-human albumin, from Scottish Antibody Production Unit, Law Hospital, Carluke, turbidity was measured using a Hitachi, 911 Discrete analyser, Boehringer Mannheim (UK), Sensitivity was 7 mg/l and C.V.= 2.50%.

2.6.4.2 Albumin in plasma

Principle

Albumin binds with bromocresol green to produce a blue complex which can be measured at 600 nm. The resulting absorbance from samples was compared with that of a standard and the concentration calculated.. (Albumin reagent kit – Olympus Optical Co Ltd.), Cat No 66001 was used and analysed using a multichannel automated analyser Olympus AV 5200, Sensitivity was 16 g/l the C.V. of the assay is 0.57 – 0.91% repsectively (Doumas et al 1971).

2.6.4.3 Fasting blood sugar in plasma

Principle

The glucose is determined after conversion to gluconate-6-phosphate with hexokinase and glucose-6-phosphate-dehydrogenase in the presence of ATP and NAD^+. The increase in absorbance of NADH at 340 nm is proportional to the glucose concentration.

$$\textit{Glucose} + \textit{ATP} \xrightarrow{HK} \textit{Glucose-6-phosphate} + \textit{ADP}$$

$$\textit{Glucose-6-phospate} + NAD^+ \xrightarrow{G6P\text{-}DH} \textit{Gluconate-6-p} + \textit{NADH} + H^+$$

Fluoride oxalate used as a blood anticoagulant. Glucose reagent kits were used prepared from Olympus Optical Co., Ltd., Cat No 066006 and measured by using an Olympus AV 5200, The C.V. of the assay is 1.9% (Teuscher et al 1971).

2.6.4.4 Urea in plasma

Principle

Urea is hydrolysed to ammonia and carbon dioxide by the catalytic action of urease. The ammonia serves to aminate 2 α-ketoglutarate to glutamate with the concurrent oxidation of NADH to NAD in the reaction catalysed by glutamate dehydrogenase. The disappearance of NADH is monitored by the rate of decrease in absorbance at 340 nm. Reagents and kit prepared from Olympus

Optical Co Ltd, Cat No 66048 and measured by using an Olympus AV 5200 analyser. The C.V. of the assay is 1.08% (Olympus insert).

2.6.4.5 Bilirubin in plasma

Principle

Indirect albumin bound bilirubin was released by a caffeine buffer. The total bilirubin reacts with diazotised sulphanilic acid to give a red azo dye. The colour produced was measured and the concentration calculated. Reagent prepared from Randex Lab Ltd., Ardmore, Crumlin, Co Antrim, Cat No OBR426, 03C and 04C. Bilirubin was measured using an Olympus Av 5200 Olympus Otpical Co Ltd. The C.V. of the assay is 0.86% and sensitivity was 5 μmol/l (Sherlock 1951).

2.6.4.6 Creatinine in plasma

Principle

Creatinine forms a yellow-orange colour with alkaline picrate solution. The increase in colour over a fixed period of time was measured photometrically, the increase in colour being directly proportional to the concentration of creatinine in the sample, was measured using an Olympus AV 5200 analyser, Olympus Optical Co Ltd, Boehringer Mannheim Jaffe cretainine, reagent kit, Cat No 1040847. The C.V. of the assay is 3.67%, 1.43% and 1% in low medium and high concentrations and sensitivity was to 30 μmol/l (Bartels et al 1972).

2.6.4.7 Total protein in plasma

Principle

Protein forms a purple complex when treated with biuret reagent (copper sulphate-alkaline tartrate-iodide) (Tietz 1987a). The amount of colour produced was measured at 540 nm. The resulting absorbancies is compared with that of a standard and the concentration calculated.

Total protein reagent kit was purchased from Olympus Optical Co Ltd., Cat No 66014 and measured by using an Olympus AV 5200. The C.V. of the assay is 1.12%, 0.96% and 0.96% for low, medium and high concentrations and sensitivity is 16 g/l. Globulin (Glob) was calculated by subtracting albumin (alb) from total protein (TP), Glob = (TP – Alb).

2.6.4.8 Alkaline phosphatase in plasma

Principle

$$\textit{P-nitrophenyl phosphate} + H_2O \xrightarrow{AP} \textit{phospate} + \textit{p-nitrophenol}$$

The concentration of PNP is measured spectrophotometrically and is proportionally to the enzyme activity.

Alkaline phosphatase reagent kit was purchased from Olympus Optical Co Ltd, Cat No 066020 and 066021 and was measured by using an Olympus AV 5200.

The C.V. of the assay is 2.78%, 4.9% and 3.6% for low, medium and high concentrations and sensitivity is 5 U/L (Klin 1972).

2.6.4.9 ALT (Alanine Aminotransferase) in plasma

Principle

$$\alpha\text{-oxoglutarate} + \text{L-alanine} \xrightarrow{GPT} \text{L-glutamate} + \text{pyrurate}$$

$$\text{Pyrurate} + NADH + H^{+} \xrightarrow{LDH} \text{L-lactate} + NAD^{+}$$

The decrease in NADH concentration is monitored spectrophotometrically at 340 nm and is proportional to the ALT activity, and was measured using ALT kit, Boehringer Mannheim (UK) Ltd, Cat No RI 360205 and R2 127799. The C.V. of the assay is 5.1%, 1.7% and 1.5% for low, medium and high concentrations and sensitivity is 5 U/l (Kennedy et al 1976).

2.6.4.10 AST (Aspartate Aminotransferase) in plasma

Principle

$$\alpha\text{-oxoglutarate} + \text{L-aspartate} \xrightarrow{GOT} \text{L-glutamate} + \text{oxaloacetate}$$

$$\text{Oxaloacetate} + NADH + H^{+} \xrightarrow{MDH} \text{L-malate} + NAD$$

Measurements were made using AST reagent kit, Boehringer Mannheim (UK) Ltd, Cat No RI 360183 and R2 127764. The C.V. of the assay is 3.5%, 2.4% and 1.9% for low, medium and high concentrations and sensitivity was to 5 U/l (Thefeld 1974).

2.6.4.11 Urate in plasma

Principle

$$\text{Uric acid} + 2\,H_2O + O_2 \xrightarrow{\text{uricase}} \text{allantoin} + CO_2 + H_2O_2$$

$$2\,H_2O_2 + H^+ + TOOS^+ + \text{4-aminophenazone} \xrightarrow{\text{POD}} \text{quinone di-imine dye} + 4\,H_2O$$

The concentration of the oxidised dye is measured spectrophotometrically and is proportional to the concentration of urate in the sample.

Measurements were made using an Olympus AV 5200 analyser, Olympus Optical Co Ltd. Uric acid reagent kts prepared from Boehringer Mannheim (UK) Ltd, Cat No RI 661 884 and R2 1661 892. The C.V. of the assay is 0.97% and sensitivity is 10 μmo/l (Thefield et al 1973).

2.6.4.12 Urine creatinine

Principle

Creatinine forms a red pigment with alkaline picrate solution. The colour develops slowly. The rate of increase in absorbance is directly proportional to the concentration of creatinine in the sample.

Measurements were made using Hi Co creatinine test kit, Boehringer Mannheim (UK) Ltd, Cat No 1040847 and measured by a Hitachi 911 Discrete analyser. The C.V. of the assay is 2% at 2000 μmol/l and sensitivity is 20 μmol/l (Bartels et al 1972).

2.6.4.13 Fructosamine

Principle

Determination of non enzymatic glycated protein (fructosamine) in plasma was based on the ability of ketoamines to reduce nitroblue tetrazolium in alkaline solution. Fructosamine reagent kits (Roche art # 42388) was used and measured photometrically at 500 nm using automated analyser (Roch Cobas Mira Discrete Analyser). Range = 205-285 μmol/l (non diabetic adult, n = 555) and 228 – 563 μmol/l for diabetic patients and C.V. = < 3%. Sensitivity was 5.72 μmol/l. (Johnson et al 1983; Schleicher & Vogt 1990).

2.6.5 Lipids and lipoproteins measurements

2.6.5.1 Cholesterol in plasma

Principle

Cholesterol was measured by enzymatic procedures. The intitial reaction step being (a) the hydrolysis of cholesterol esters to release free cholesterol and (b) a subsequent oxidation step to produce hydrogen peroxide which was quantified by (c) the formation of a coloured oxidation product quinoneimine which absorbs light at 505 nm.

$$\text{(a)}\quad \textit{cholesterol esters} + H_2O \xrightarrow{\textit{cholesterol esterase}} \textit{cholesterol} + \textit{RCOOH}$$

$$\text{(b)}\quad \textit{cholesterol} + O_2 \xrightarrow{\textit{cholesterol oxidase}} \textit{4-cholestenone} + H_2O_2$$

$$\text{(c)}\quad 2H_2O_2 + \textit{4-aminophenazone} + \textit{phenol} \xrightarrow{\textit{POD}} \textit{4-(p-benzoquinone-mono-imino)-phenazone} + H_2O$$

Sensitivity was 0.1 mmol/l using a cholesterol reagents kit Boehringer Mannheim, Cat No 1489437. Samples were measured by Hitachi 717 analyser (Boehringer Mannheim) (Manual of laboratory operations 1974) (Siedel et al 1983).

2.6.5.2 Triglyceride in plasma

Principle

Triglyceride was measured by enzymatic hydrolysis with subsequent enzymatic determination of the liberated glycerol by colorimetry (a) hydrolysis of triglycerides to form glycerol is achieved by lipase. Glycerol produced by hydrolysis was assayed by a coupled-enzyme approach. First step was (b) conversion of glycerol to glycerol-3-phospate by glycerol kinase (GK) and (c) glycerol phosphate oxidase (GPO) produces hydrogen peroxide which was reacted with (d) peroxidase to give a red quineimine dye which absorbs light at 505 nm.

$$\text{(a)}\quad TG + 3H_2O \xrightarrow{\text{lipase}} \text{glycerol} + 3\ R\ COOH$$

$$\text{(b)}\quad \text{glycerol} + ATP \xrightarrow{GK} \text{glycerol-3-phosphate} + ADP$$

$$\text{(c)}\quad \text{glycerol-3-phosphate} + O_2 \xrightarrow{GPO} \text{dihydroxyacetone phosphate} + H_2O_2$$

$$\text{(d)}\quad H_2O_2 + \text{4-aminophenazone} + \text{4-chlorophenol} \xrightarrow{\text{peroxidase}} \text{4-(p-benzoquinone-mono-imino)-phenazone} + 2H_2O + Hcl$$

Sensitivity was 0.05 mmol/l. Reagents were prepared from Boehringer Mannheim reagent kit, Cat No 1488899 used and measured by a Hitachi 717 analyser (Boehringer Mannheim) (Trinder et al 1969).

2.6.5.3 Full lipoprotein analysis (LDL, HDL and VLDL) in plasma

Principle

Lipoproteins can be selectively precipitated by adding combinations of sulphated polysaccharides and divalent cations to plasma. 5 ml of plasma in a thermoplastic ultra centrifuge tube was overlayed with normal saline, density 1.006 kg/l. The tube was then sealed with a cap and ultracentrifuge (Beckman, USA) at 35000 rpm at $4^{o}C$ for 16 hours after which two fractions were obtained, a "top" fraction containing VLDL and a "bottom" fraction containing HDL and LDL. The LDL component precipitated by using heparin and manganous chloride leaving the HDL in solution (Manual of laboratory operations 1974).

2.7 Test diets

2.7.1 Design of low and high flavonoids diet for flavonoid study in diabetic patient

Ten diabetic patients followed a low flavonoids diet for a 28 days study (according to the dietetic instruction). They were randomly assigned to receive either high or low flavonoids diet for 14 day periods. After this they received

the alternative low or high flavonoids diet for 14 days, in a crossover design. Each subject thus acted as their own control.

2.7.2 Dietary Intervention

2.7.2.1 Low flavonol diet (28 days):

Patients were advised by a state-registered dietitian to avoid eating high flavonol foods including certain drinks (red wine, fruit juices of all varieties, tea), fruit; especially apples, oranges, strawberries, grapes, berries, currants and sultanas, vegetables and salads, particularly onion, garlic, shallots, tomatoes, lettuce, celery, beans, parsley, cabbage leaves, peppers (red, green, yellow) and nuts (walnut, hazelnut, peanut). Advice was given to maintain normal energy intake, to avoid weight change.

2.7.2.2 High flavonol diet: 14 days supplements in addition to low flavonol diet

Introduction

According to published data, onions have a particularly high flavonoid content (Herrman et al 1988, Hertog et al 1992, Dorant et al 1994, Hollman et al 1996a and Crozier et al 1997a). Also teas are beverages with very high flavonoid content (Herrman et al 1988 , Shahidi et al 1992, Xie et al 1993, Hertog et al 1993b, Rice-Evans et al 1995 and 1996,and Guo et al 1996). Hertog suggested that the most important sources of total flavonoid intake in the Netherlands are onions (29%) and tea (48%) (Hertog et al 1993a).

Following collaborative studies between the Department of Human Nutrition and the Division of Biochemistry and Molecular Biology of University of Glasgow, we concluded that purified flavonoids were not adequately absorbed. Onion and tea were chosen as diet supplements for flavonoids study on diabetic patients, on the basis of high flavonoid contents. It was recognised that these foods also contain other potentially bioactive compounds, notably catechins and epicatechins in tea. At the time of study design, there was no evidence for absorption in humans.

Onion Supplement: 400 g yellow English onions, without dry skin, were chopped with a food processor into medium slices and fried lightly for 1.5 min with 20 g olive oil (extra virgin). Immediately after cooking the fried onions were divided into three freezer bags (to be consumed in 133 g bags as three meals daily) and frozen for storage at –20°C before eating.

Onion, tomato ketchup and herb supplement: 400 g yellow English onions, without dry skin, were chopped with a food processor into medium slices and lightly fried with for 1.5 min with 20 g olive oil (extra virgin), 20 g tomato ketchup (Heinz) and 1g Italian seasoning herbs (Safeway) and consumed in three equal mealtime supplements.

Tea Supplement: six mugs (250 ml) of tea/day (Typhoo tea bag, 5 minutes infusion).

Fried onion bags for consumption during the 14 days on high flavonoid diet were provided to subjects in deliveries every 4 days (each of 26 x 133 g bags). One subject used decaffeinated tea at his own request. During these periods they continued to avoid eating other high flavonoids foods (according to the low flavonoid diet instruction).

2.7.2.3 Composition of supplements: Flavonols were measured by HPLC and the results shown in **Table 6.2.** The onion and tomato ketchup supplements were analysed for their antioxidant vitamin contents. Vitamin C content was measured using a colorometric method and was 3.6 mg/100 g while vitamins E and A were both undetectable (< 2 mg/100 g) using HPLC methods. Tea did not contain any of these vitamins.

2.7.3 Four days food diary records

Four days before start of the flavonoid study (including weekend days) all subjects completed a 4 days food diary. Instruction for completing 4 -days food and drink dairy by picture and scale was explained to all subjects by a dietitian (Irene Kelly, MSc, SRD). Diabetic subjects recorded everything that they ate and drank, and included a description and weight of each individual food and drink item and how much food was left in plate or cup.

Energy and nutrient intake for subject was calculated by a state registered research dietitian. This information was then analysed by COMP-EAT , v. 4.0

software (Nutrition System, Carlson Bengslon consultant Ltd., England, UK). This programme is based on the data in McCance & Widdowson Composition of Foods 8th edition food composition table (McCance and Widdowson et al 1991).

2.7.4 Determination of antioxidant vitamins in the test diet

2.7.4.1 Determination of vitamins A and E in test diet

Both vitamins A (retionol) and E (alpha-tocopherol) were determined by (HPLC) technique. In order to free the vitamins from the food matrix and eliminate bulk components such as triglycerides, the food samples were saponified.

Owing to sensitivity of vitamins A and E to oxidation and to destruction by light, all determinations were carried out in the absence of oxygen (under nitrogen gas) and shielded from light using amber coloured actinic glass.

The vitamins were detected and quantified by HPLC using an ACS 352 HPLC pump running isocratically with methanol and water as mobile phase (2ml/min) and a Cecil CE 1400 refractive index detector. Separation was achieved on a 25 cm x 4.6 mm Techsphere 5ODS column (HPLC Technology Ltd, Warrington, UK) (Pearson's composition and analysis of foods, 1991).

2.7.4.2 Determination of vitamin C in test diet

A 25% of the solid food sample was prepared in triple distilled water. Fifty to one hundred millilitres of the prepared sample was added to equal volume of extraction solution and mixed thoroughly. An aliquot containing about 2 mg of ascorbic acid was titrated with standard indophenol solution and corrected for blank using an equivalent amount of extraction solution. Extraction solution – dissolve 15 g of phosphoric acid in 40 ml acetic acid and 200 ml water, dilute to 500 ml and filter.

Standard solution – dissolve 0.05 g ascorbic acid in 45 ml of the extraction solution and make up to 50 ml. Prepare immediately before use. Indophenol standard solution – dissolve with shaking 0.05 g of 2,6-dicholorophenolindophenol (sodium salt) in 50 ml water containing 42 mg sodium bicarbonate. Dilute to 200 ml with water. Filter. Standardise by titration against 2 ml of standard ascorbic acid solution added to 5 ml of the extraction solution.Indicator – dissolve 0.1 g thymol blue in 10.75 ml 0.02N sodium hydroxide solution, dilute to 250 ml with water (Pearson's composition and analysis of foods, 1991).

2.8 Statistical methods

2.8.1 Statistical methods in the comet assay (Chapter 3)

Linear regression analysis of damage score versus log concentration of antioxidant was used to assess the dose-dependency of the protective effect. The

concentration that would reduce the damage score obtained in the absence of the flavonoid by 50% (ED50) was estimated from the regression line (in some cases by extrapolation) as a comparative measure of efficacy. Separate comparisons of the antioxidant activity of different agents were performed at each concentration, using one-way ANOVA together with Tukey's method for all pairwise comparisons between flavonoids, and Dunnett's method for comparing all flavonoids with vitamin C. The significance levels reported refer to error rates for each family of comparisons.

The effect of quercetin and vitamin C in combination was analysed by two-way ANOVA. For free flavonoids, the relationship between the number of hydroxyl groups in the structure of the agents and the degree of protection against DNA damage was assessed by linear regression analysis. Statistical significance in all cases was assessed at the 5 per cent level.

2.8.2 Statistical methods in the TEAC assay (Chapter 4)

Comparisons between the antioxidant activity of different agents (flavonoids, polyphenols and vitamin C) were performed by using one-way ANOVA with Tukey's method of analysis. Associations between TEAC and concentration of rutin and quercetin in human plasma and between TEAC and the number of OH-groups in each flavonoid were tested by calculating the Pearson correlation coefficient (R) and the coefficient of determination (R^2), which was expressed as a percentage (i.e. R^2 gives the percentage variance explained by the independent

variable). The significance of association between pairs of variables was determined by linear regression.

2.8.3 Statistical methods in the response of diabetic patients with high flavonoids diet (Chapter 6)

Appropriate parametric or non-parametric analyses for paired data were used, to compare data at the ends of low and high flavonol-diet periods. Wilcoxon's test was used for all comparisons except SCGE and endonuclease III, whose data appeared to be normally distributed and paired t-tests were employed. All data are presented as mean $\pm$ standard error of mean. A p value <0.05 is regarded as statistically significant.

2.8.4 Statistical methods in the prediction of dietary flavonol consumption from fasting plasma concentration or urinary excretion (Chapter 7)

The data on total flavonoids, and on quercetin combine both free (aglycone) and conjugated forms. Linear regression analysis of plasma and urine quercetin and flavonoids versus flavonoids and quercetin diet were used to assess the relation between the factors. Statistical significance in all cases was assessed at the 5% level. The equations of the regression lines were used to estimate flavonol and quercetin intakes given in tables 7.3 & 7.4.

CHAPTER 3: Protection from pre treatment with various flavonoids and vitamin C against oxygen radical generated DNA damage in *ex vivo* lymphocytes

(paper accepted for publication in American Journal of Clinical Nutrition, co-authors W Angerson, MEJ Lean)

Abstract

This study assessed the antioxidant potencies of several widespread dietary flavonoids, across a range of concentrations and compared to vitamin C as a positive control. The antioxidant effects of pre-treatment with flavonoids and vitamin C, in standardised concentrations (7.6, 23.2, 93 and 279.4 μmol/L), on oxygen-radical generated DNA damage from hydrogen peroxide (100 μmol/L) in human lymphocytes were examined using the single-cell gel electrophoresis assay (SCGE assay or "comet assay").

Pre-treatment with all flavonoids and vitamin C produced dose-dependent reductions in oxidative DNA damage. At a concentration of 279 μmol/L, they were ranked in decreasing order of potency as follows: luteolin (9% of damage from unopposed hydrogen peroxide), myricetin (10%), quercetin (22%), kaempferol (32%), quercitrin (quercetin-3-L-rhamnoside) (45%), apigenin

(59%), quercetin-3-glucoside (62%), rutin (quercetin-3β D-rutinoside), (83%) and vitamin C (78% of damage).

The protection of vitamin C against DNA damage at this concentration was significantly less than that of all the flavonoids except apigenin, quercetin-3-glucoside and rutin. Ranking was similar using estimated ED50 (concentration to produce 50% protection). The protective effect of quercetin and vitamin C at a concentration of 23.2 μmol/L was found to be additive (quercetin 71% of maximal DNA damage from unopposed hydrogen peroxide, vitamin C 83% , both in combination 62%). These data suggest that the free flavonoids are more protective than the conjugated flavonoids (e.g. quercetin versus its conjugate quercetin-3-glucoside, $p<0.001$). They are also consistent with the hypothesis than antioxidant activity of free flavonoids is related to the number of hydroxyl groups.

3.1 Introduction

Flavonoids were initially considered to be non-nutrients i.e. substances without any nutritive value for humans. However in 1936 Szent Gyorgi had shown that two flavonoids derived from citrus fruits decreased capillary fragility and permeability in humans (Rusznya'k & Gyorgyi et al 1936).

The aim of this study was to determine the antioxidant capacities of various flavonoids and vitamin C against the oxidative DNA damage produced in ex vivo human lymphocytes by hydrogen peroxide.

The antioxidant effect of flavonoids

Quercetin, myricetin, kaempferol, rutin and vitamin C are powerful antioxidants in the oxidation of low-density lipoprotein and provide a possible mechanism for the beneficial epidemiological effect of dietary fruit and vegetables on heart disease (Vinson et al 1995). Evidence comes from several sources. Using a high temperature incubation method Mehta first reported that quercetin has an antioxidant action (Mehta et al 1958). Hudson found that quercetin and luteolin have good primary antioxidant activity in the stability of lard, using the 100°C method (Hudson et al 1983). A study using *uv*-induced oxidation of LDL (Negre et al 1995) showed that rutin, a polyphenolic flavonoid, vitamin C and vitamin E were able to inhibit the peroxidation of LDL and their subsequent cytotoxicity.

Quercetin (3',5,7, 3',4' pentahydroxyflavon) prevents oxidation of LDL by macrophages in vitro by reducing the formation of free radicals (De Whalley et al 1990; Chen et al 1996) and dietary intake of quercetin estimated from dietary records is inversely related to coronary heart disease mortality (Hollman et al 1995). A number of more recent studies, using superoxide assays, have

confirmed that quercetin is a strong antioxidant, and most flavonoids show antioxidant activity (Yukiko et al 1994; Laughton et al 1989).

Single cell gel electrophoresis (the comet assay) is a sensitive and rapid method for the detection of DNA damage at the individual cell level (McKelvey-Martin et al 1993; Fairbairn et al 1995) and specifically for detecting oxidative DNA strand breaks (Duthie 1997; Collins et al 1995 & 1997; Singh et al 1988). It is considered a useful tool for investigating issues related to oxidative stress in human lymphocytes (Green et al 1992) but has not previously been used with flavonoids. We therefore used the comet assay to evaluate the antioxidant capacity of some major dietary flavonoids, with vitamin C as a positive control.

Material and methods

The study design and methods are explained in detail in Chapter 2 part 2.2.

Statistical methods

Explained in detail in Chapter 2 part 2.8.1.

3.2 Results

All the flavonoids and vitamin C produced dose-dependent reductions in oxidative DNA damage as assessed by linear regression analysis of the log-dose

response curves. The coefficient of variation was 19% for duplicate assessments of DNA damage and 20% for repeated identical experiments.

The effects of flavonoids and vitamin C treatment against oxidative DNA damage in human lymphocytes in the comet assay are shown in **Tables 3.1.A to 3.1.I.**

Examples of dose-response curves are shown in **Figure 3.1.** The concentrations that would produce a 50% reduction in DNA damage as estimated from the regression equations were, in decreasing order of efficacy: quercetin 47 μmol/L, luteolin 51μmol/L, myricetin 64 μmol/L, kaempferol 104 μmol/L, quercitrin 288 μmol/L, quercetin-3-glucoside 984 μmol/L, apigenin 1.5 mmol/L, rutin 43 mmol/L and vitamin C 233 mmol/L (**Figure 3.2**). The values for quercitrin, quercetin-3-glucoside, apigenin, rutin and vitamin C were derived by extrapolation of the dose-response curves and should be regarded as approximate measures of relative efficacy rather than true ED50 values.

The results of all pairwise comparisons of the antioxidant effect of the nine agents at a concentration of 279 μmol/L are shown in **Table 3.2.** Although there are minor differences in the ranking of the agents as compared with the ED50 values, none of these relate to any statistically significant differences. The four most potent agents studied (luteolin, myricetin, quercetin and kaempferol) are all members of the group of free flavonoids (which also includes apigenin) and in

most pairwise comparisons were significantly more effective than the conjugated flavonoids (quercitrin, quercetin-3-glucoside and rutin). Vitamin C was significantly less potent than luteolin, myricetin, quercetin, kaempferol and quercitrin. It did not differ significantly in efficacy from quercetin-3-glucoside, apigenin and rutin. At lower concentrations, there were fewer statistically significant differences between the agents. Rutin was consistently the weakest antioxidant of all the flavonoids tested, and vitamin C also remained less effective than the majority of other agents. Quercetin was consistently one of the most potent.

In a single experiment, the antioxidant effects of vitamin C, quercetin, and both these agents combined were assessed at a fixed relatively low concentration of 23.2 μmol/L. The results are shown in **Figure 3.3**. Quercetin alone reduced oxidative DNA damage by 29% relative to untreated control cells, while vitamin C alone reduced damage by 17% . The two agents combined reduced damage by 38%. Two-way ANOVA showed that the treatment effects of both agents were significant (quercetin, F=18.49, df=1,16, p<0.001; vitamin C, F=5.08, df=1,16, p=0.04) and that the interaction between them was insignificant. The analysis therefore suggests that the protective effects of these agents at the doses investigated were additive.

The mean damage score for the five free flavonoids at a concentration of 279 μmol/L is plotted against the number of hydroxyl groups in the structure of the

molecule in **Figure 3.4**. There was a negative correlation between these variables, although for the small number of agents studied it failed to reach statistical significance (r=-0.60, n=5, p=0.17).

3.3 Discussion

The results of the present study indicate that the aglycones quercetin, luteolin, myricetin (**Figure 3.5**) and kaempferol have a greater antioxidative capacity than the conjugate flavonoids, such as quercetin-3-glucoside, quercitrin and rutin (**Figure 3.6**). This is in agreement with the results of several other studies using a wide range of methods for assessing antioxidant activity, as shown in **Table 3.3**. In our study, apigenin was the least potent of the free flavonoids, and this is in agreement with the previous studies (Galvez et al 1995; Vinson et al 1995). Also, Chen et al reported that apigenin demonstrated no antioxidant activity in rape seed oil heated at 105^{o}C (Chen et al 1996).

The position and number of hydroxyl groups has an important role in antioxidant activity (Chen et al 1996; Shimoi et al 1994). In our study, at a concentration of 279 μmol/L, the protection of myricetin, quercetin, kaempferol and apigenin against DNA damage would be consistent with a relationship to the number of hydroxyl groups. For apigenin, the three hydroxyl groups at positions 5, 7 and 4' were associated with a small but definite antioxidant effect, whereas others have found this agent to provide no protection against oxidative damage (Chen et al

1996). Kaempferol, with an additional hydroxyl group at position 3, was more protective than apigenin, and quercetin and myricetin, with further groups at the 3' and 5' positions, were still more effective (**Figure 3.7**). The antioxidant activity of quercetin involves hydrogen-atom donation to peroxy radicals, thus terminating the chain radical reaction (Torel et al 1986). Shahidi has shown flavonoids are excellent hydrogen donors and those with a 3',4' dihydroxy configuration such as quercetin possess strong antioxidant activity (Shahidi et al 1992). Luteolin, with a similar number of hydroxyl groups to kaempferol, was significantly more effective in the present study, as others have also reported (Galvez et al 1995; Shimoi et al 1994). This may be because the hydroxyl group at the 3' position in luteolin confers greater antioxidant activity than the group at the 3 position in kaempferol.

The single cell gel electrophoresis (comet) assay has been explored as a potential tool for detecting the antioxidant effect of foods or nutrients. Supplementation with vitamin C (100 mg/d), vitamin E (280 mg/d) and beta carotene (25 mg/d) for 20 wk significantly decreased endogenous oxidative DNA damage in human lymphocytes (Duthie et al 1996). Hartman et al used the comet assay to study DNA damage in peripheral white blood cells of humans after exhaustive exercise and reported that vitamin E supplementation prevents exercise induced DNA damage (Hartman et al 1995). Green et al found a reduction in radiation-induced DNA damage following vitamin C ingestion (35 mg/kg) (Green et al 1994). The effect of various antioxidants on oxygen-radical-generated DNA damage in

human lymphocytes has been investigated by using the comet assay. There were small protective effects of vitamin C at low doses of 40 μmol/L and no protection at high doses of 5 mmol/L. Trolox (water soluble analogue of vitamin E) produced no effect against DNA damage (Anderson et al 1994).

The present study showed that the SCGE or "comet assay" can be used to give reproducible results in estimating the extent of DNA damage to human lymphocytes. It thus proved possible to rank the potency of the antioxidant agents tested with high confidence. Vitamin C, well recognised as a dietary antioxidant, and the concentrations of vitamin C tested were chosen to span the normal plasma concentrations 34-114 μmol/L (Tietz 1987). Much higher concentrations 15 mg/100 mL are found in leucocytes including lymphocytes which concentrate vitamin C to levels 14 fold greater than plasma (Tietz 1987; Levine et al 1996). Intracellular concentrations were not measured in the present study. Fruit and vegetables provide about 60% of total vitamin C intake and these foods are likely to contribute other antioxidants including flavonoids. At equimolar concentrations the results demonstrate very clearly a greater antioxidant potency from most of the flavonoids tested than from vitamin C. The results also showed that the effects of quercetin, one of the most potently antioxidant flavonoids, and vitamin C, could be additive when cells were pretreated with both at concentrations of 23.2 μmol/L.

The conclusions from these in vitro experiments must remain tentative, until more is known about the absorption, distribution, metabolism and biological effects of flavonoids within the body. The present study supports the possibility that other bioactive compounds such as flavonoids, which are known to have appropriate actions (**Table 3.3**), may be important factors for health which coexist with the more familiar antioxidant vitamins. The importance of quercetin, one of the most widespread and also most potent antioxidant flavonol, is underlined by the results of the present study.

TABLE 3.1.A The effect of *Kaempferol* pre-treatment against oxidative DNA damage in human lymphocytes in the comet assay (SCGE assay).

Treatment	Percentage of the cells showing different grades of DNA damage[a]					
	No damage (<5 %)	Low damage (5-25 %)	Medium damage (25-45 %)	High damage (45-70 %)	Very High damage (>70 %)	Total score (out of 400)
100 µmol/L H_2O_2 *(no kaempferol)*	14.0 ±4.6	22.0± 2.5	10.4±2.2	13.2±2.2	40.3±3.0	243.6±12.7
Control [b]	86.3±2.1	12.3±1.9	0.5±0.3	0.7±0.3	0.2±0.2	16.0±2.5
7.6 µmol/L *Kaempferol* + 100 µm H_2O_2	27.4 ±6.2	23.2± 3.7	7.5±1.8	11.3±1.4	30.6±3.1	194.5±15.2
Control	88.6±3.1	9.8±2.9	0.8 ± 0.4	0.5±0.2	0.2±0.2	13.7±3.4
23.3 µmol/L *Kaempferol*+ 100 µm H_2O_2	33.0 ± 3.4	23.8± 3.9	6.2±1.0	11.5±1.0	25.6±2.5	173.7±8.2
Control	85.2±2.7	12.2±2.8	1.3±0.5	0.8±0.3	0.5±0.3	19.3±3.0
93 µmol/L *Kaempferol* + 100 µm H_2O_2	47.8 ± 4.3	17.8±3.0	3.2±1.0	8.5±1.3	22.8± 2.4	140.9±10.2
Control	94.3±1.6	4.8±1.6	0.5±0.2	0.3±0.2	0.0±0.0	6.8±1.4
279.4 µmol/L *Kaempferol* +100 µmH_2O_2	68.8 ± 2.4	10.2±2.1	4.5±0.9	5.6±1.0	11.0±1.5	78.6±8.2
Control	94.2±1.3	4.8±1.5	0.3±0.2	0.7±0.4	0.0±0.0	7.5±1.2

[a] Values represent duplicates from six experiment s (means ± SEM)

[b] Control samples with no H_2O_2

TABLE 3.1.B The effect of *quercetin* treatment against oxidative DNA damage in human lymphocytes in the comet assay (SCGE assay)

Treatment	Percentage of the cells showing different grades of DNA damage[a]					
	No damage (<5%)	Low damage (5-25%)	Medium damage (25-45%)	High damage (45-70%)	Very High damage (>70%)	Total Score (out of 400)
100 µmol/L H_2O_2 *(no quercetin)*	16.2 ± 3.6	10.5± 2.2	5.3±1.9	14.5±1.7	53.5±4.5	278.7±15.6
Control [b]	91.3±2.3	3.7±0.9	3.0±1.2	1.3±0.6	0.7±0.7	16.3±5.8
7.6 µm ol/L*quercetin* + 100 µm H_2O_2	26.5 ± 3.7	12.0± 2.4	7.3±1.3	23.0±2.0	31.2±4.2	216.3±12.6
Control	84.7±4.8	6.7±1.2	2.7 ± 0.9	5.3±3	0.7±0.7	30.7±12.7
23.3 µmol/L *quercetin* + 100 µm H_2O_2	36.8 ± 4.3	12.3± 2.3	7.8±1.4	19.7±4.1	23.3±4.3	180.3±15.2
Control	92.0±5.1	6.3±5.4	0.7±0.3	1.0±0.6	0.0±0.0	10.7±4.6
93 µmol/L *quercetin* + 100 µm H_2O_2	48.8 ± 3.6	15.0±3.0	7.7±1.0	24.0±4.7	4.2± 1.4	119.0±15.11
Control	97.0±2.0	2.7±1.7	0.3±0.3	0.0±0.0	0.0±0.0	3.3±4.0
279.4 µmol/L *quercetin* +100 µmH_2O_2	63.2 ± 2.8	20.5±2.3	9.0±2.1	7.2±2.3	0.2±0.2	60.7±8.3
Control	94.3±2.3	6.0±2.8	0.0±0.0	1.3±1.3	0.0±0.0	8.3±3.3

[a] Values represent duplicates from three experiment (means ± SEM) [b] Control sampels with no H_2O_2

TABLE 3.1.C The effect of *Myricetin* treatment against oxidative DNA damage in human lymphocytes in the comet assay (SCGE assay)

Treatment	Percentage of the cells showing different grades of DNA damage[a]					
	No damage (<5%)	Low damage (5-25%)	Medium damage (25-45%)	High damage (45-70%)	Very High damage (>70%)	Total score (out of 400)
100 µmol/L H_2O_2 (*no Myricetin*)	17.3±3.6	20.3±2.1	8.8±1.4	16.0±1.4	37.5±1.6	236.0±9.6
Control [b]	89.0±6.7	9.0±5.1	1.0±1.0	0.7±0.7	0.3±0.3	14.3±8.6
7.6 µmol/L (*Myricetin*)+ 100 µm H_2O_2	25.2±3.7	22.2±1.7	16.3±3.9	12.0±1.1	24.3±1.9	189.7±9.8
Control	89.0±2.6	7.3±2.2	2.7±0.7	1.0±0.6	0.0±0.0	15.7±3.8
23.3 µmol/L (*Myricetin*) + 100 µm H_2O_2	30.5±4.9	24.7±4.5	7.7±2.1	14.3±2.2	22.8±2.9	174.8±10.3
Control	86.0±4.0	12.0±4.6	1.7±0.9	0.3±0.3	0.0±0.0	16.3±3.8
93 µmol/L (*Myricetin*) + 100 µm H_2O_2	40.2 ±4.3	23.8±2.2	8.2±1.7	16.2±3.3	11.7±1.7	135.3±14.9
Control	85.0±3.6	13.0±2.9	2.0±1.2	0.0±0.0	0.0±0.0	17.0±4.5
279.4 µmol/L (*Myricetin*) +100 µmlH_2O_2	79.2±6.2	17.8±6.1	2.33±0.67	0.67±0.21	0.0±0.0	24.5±6.3
Control	89.0±8.5	10.0±8.0	0.3±0.3	0.7±0.3	0.0±0.0	12.7±9.3

[a] Values represent duplicates from three experiment (means ± SEM). [b] Control samples with no H_2O_2

TABLE 3.1.D The effect of *Luteolin* treatment against oxidative DNA damage in human lymphocytes in the comet assay (SCGE assay)

Treatment	Percentage of the cells showing different grads of DNA damage[a]					
	No damage (<5%)	Low damage (5-25%)	Medium damage (25-45%)	High damage (45-70%)	Very High damage (>70%)	Total score (out of score)
100 µmol/L H_2O_2 (*no Luteolin*)	19.5±2.8	21.0±2.4	9.3±1.5	17.0±1.1	33.2±5.0	223.3±16.5
Control [b]	89.7±2.3	9.7±2.0	0.3±0.3	0.3±0.3	0.0±0.0	11.3±2.7
7.6 µmol/L (*Luteolin*)+ 100 µm H_2O_2	26.7±5.3	17.0±3.2	7.8±2.1	18.8±1.9	29.7±6.4	207.8±21.6
Control	91.0±1.0	8.7±0.9	0.3 ± 0.3	0.0±0.0	0.0±0.0	9.3±1.2
23.3 µmol/L (*Luteolin*) + 100 µm H_2O_2	37.7± 3.4	9.8± 2.7	8.3±0.6	28.5±4.2	15.7±3.3	174.7±14.5
Control	94.0±0.6	5.7±0.3	0.3±0.3	0.0±0.0	0.0±0.0	6.3±0.9
93 µmol/L (*Luteolin*) + 100 µm H_2O_2	61.2±5.0	20.2±3.4	7.3±1.9	10.7±3.0	0.8±0.5	70.2±11.1
Control	94.0±1.2	5.3±1.5	0.3±0.3	0.3±0.3	0.0±0.0	7.0±0.6
279.4 µmol/L (*Luteolin*) +100 µmH_2O_2	84.8±4.0	11.0±2.8	3.3±1.1	0.8±0.4	0.0±0.0	20.2±5.5
Control	96.7±1.2	3.3±1.2	0.0± 0.0	0.0±0.0	0.0±0.0	3.3±1.2

[a] Values represent duplicates from three experiment (means ± SEM). [b] Control samples with no H_2O_2

TABLE 3.1.E The effect of *Quercitrin* treatment against oxidative DNA damage in human lymphocytes in the comet assay (SCGE assay)

Treatment	Percentage of the cells showing different grades of DNA damage[a]					
	No damage (<5%)	Low damage (5-25%)	Medium damage (25-45%)	High damage (45-70%)	Very High damage (>70%)	Total score (out of 400)
100 µmol/L H_2O_2 (*no Quercitrin*)	24.4 ±6.7	18.9± 2.1	7.6±0.9	14.1±2.4	35.0±4.2	216.4±21.8
Control [b]	90.0±2.8	7.3±2.7	1.8±0.3	1.0±0.0	0.0±0.0	13.8±2.96
7.6 µmol/L (*Quercitrin*)+ 100 µm H_2O_2	31.2±3.8	21.8±2.2	10.3±3.6	14.7±2.5	22.0±4.2	166.2±17.7
Control	88.8±2.2	9.8±2.8	1.3 ± 0.5	0.3±0.3	0.0±0.0	13.0±1.6
23.3 µmol/L (*Quercitrin*) + 100 µm H_2O_2	34.0± 6.8	21.3± 1.8	7.5±2.2	16.8±2.5	20.3± 4.2	168.2±19.2
Control	86.6±4.3	10.8±4.1	2.0±0.5.	0.4±0.4	0.2±0.2	16.8±4.4
93 µmol/L (*Quercitrin*) + 100 µm H_2O_2	45.3±6.7	17.0±4.9	6.7±0.8	13.8±2.9	17.2±1.8	140.5±13.2
Control	90.3±4.4	9.0±4.2	0.5±0.3	0.3±0.3	0.0±0.0	10.8±4.4
279.4 µmol/L (*Quercitrin*) +100 µmH_2O_2	56.0±6.7	14.5±2.4	6.3±1.4	15.5±2.2	7.7±3.2	97.7±19.2
Control	94.7±3.0	4.7±2.3	0.7± 0.7	0.0±0.0	0.0±0.0	6.0±3.6

[a] Values represent duplicates from three experiment (means ± SEM). [b] Control samples with no H_2O_2

TABLE 3.1.F The effect of *Apigenin* treatment against oxidative DNA damage in human lymphocytes in the comet assay (SCGE assay)

Treatment	Percentage of the cells showing different grades of DNA damage[a]					
	No damage (<5%)	Low damage (5-25%)	Medium damage (25-45%)	High damage (45-70%)	Very High damage (>70%)	Total score (out of 400)
100 µmol/L H_2O_2 (*no Apigenin*)	13.8±1.4	22.5±1.6	8.2±1.1	16.7±2.1	38.8±1.4	244.2±4.5
Control [b]	81.7±6.7	13.7±7.1	4.0±2..5	0.7±0.3	0.0±0.0	23.7±6.9
7.6 µmol/L (*Apigenin*)+ 100 µm H_2O_2	26.3±3.2	23.5±1.5	7.5±1.9	15.7±2.4	27.0±1.8	191.2±7.2
Control	91.7±1.8	3.7±2.3	3.7± 2.7	1.0±0.6	0.0±0.0	11.0±2.5
23.3 µmol/L (*Apigenin*) + 100 µm H_2O_2	19.8±3.9	29.3±1.9	12.3±1.0	18.2±2.9	15.7±2.3	189.8±8.8
Control	85.3±2.9	12.7±2.0	1.7±1.2	0.3±0.3	0.0±0.0	17.0±3.6
93 µmol/L (*Apigenin*) + 100 µm H_2O_2	36.7±2.0	21.8±2.4	6.3±1.0	15.7±0.7	19.5±1.4	161.0±6.0
Control	86.0±2.9	13.3±3.2	0.3±0.3	0.3±0.3	0.0±0.0	15.0±2.3
279.4 µmol/L (*Apigenin*) +100 µmH_2O_2	40.0±2.3	24.5±2.5	6.5±2.1	10.7±1.4	18.3±2.5	143.2±5.5
Control	90.0±2.3	8.0±2.1	1.7±0.3	0.3±0.3	0.0±0.0	12.3±3.0

[a] Values represent duplicates from three experiment (means ± SEM). [b] Control samples with no H_2O_2

TABLE 3.1.G The effect of *Quercetin-3-glucoside* treatment against oxidative DNA damage in human lymphocytes in the comet assay (SCGE assay)

Treatment	Percentage of the cells showing different grades of DNA damage[a]					
	No damage (<5%)	Low damage (5-25%)	Medium damage (25-45%)	High damage (45-70%)	Very High damage (>70%)	Total score (out of 400)
100 µmol/L H_2O_2 (*no Q-3-G*)	12.7 ±3.0	16.2± 4.8	10.8±1.9	16.3±0.7	44.8±5.7	266.2±17.9
Control [b]	81.0±2.5	16.0±1.2	1.7±0.7	1.3±0.5	0.3±0.3	24.7±4.8
7.6 µmol/L (*Q-3-G*)+ 100 µm H_2O_2	16.8 ±3.1	21± 4.3	8.7±1.6	20.7±1.4	33.7±2.0	241.5±5.4
Control	80.0±4.2	14.7±2.7	2.0 ±1.5	3.3±0.8	0.0±0.0	28.7±6.1
23.3 µmol/L (*Q-3-G*) + 100 µm H_2O_2	26.7 ± 4.1	24.7± 4.5	8.3±0.5	16.7±1.1	24.5±2.1	189.3±7.7
Control	89.7±0.9	9.7±0.9	0.3±0.3	0.0±0.0	0.3±0.3	11.7±1.7
93 µmol/L (*Q-3-G*)+ 100 µm H_2O_2	39.8 ± 3.2	12.5±3.7	5.8±1.6	17.5±1.8	24.5± 2.6	174.7±10.4
Control	87.0±0.6	10.3±0.9	1.3±0.3	1.3±0.3	0.0±0.0	17.0±1.2
279.4 µmol/L (*Q-3-G*)+100 µmH_2O_2	42.8± 3.5	13.7±2.4	7.5±1.5	12.7±1.4	24.3±1.3	164.0±14.1
Control	89.7±2.0	9.3±1.9	0.0±0.0	1.0±0.6	0.0±0.0	12.3±2.7

[a] Values represent duplicates from three experiment (means ± SEM) [b] Control samples with no H_2O_2 (Q-3-G) = Quercetin-3-glucoside

TABLE 3.1.II The effect of *Vitamin C* treatment against oxidative DNA damage in human lymphocytes in the comet assay (SCGE assay)

Treatment	Percentage of the cells showing different grades of DNA damage[a]					
	No damage (<5%)	Low damage (5-25%)	Medium damage (25-45%)	High damage (45-70%)	Very High damage (>70%)	Total score (out of 400)
100 µmol/L H_2O_2 *(no vitamin C)*	18.7±2.3	19.8±3.5	7.2±1.2	15.0±1.9	39.3±2.6	236.5±6.8
Control [b]	94.0±3.0	6.0±3.0	0.0±0.0	0.0±0.0	0.0±0.0	6.0±3.0
7.6 µmol/L (*Vitamin C*)+ 100 µm H_2O_2	19.3±3.9	19.7±3.6	9.2±1.4	19.8±3.3	32.0±4.3	225.5±18.8
Control	97.5±1.5	2.5±1.5	0.0±0.0	0.0±0.0	0.0±0.0	2.5±1.5
23.3 µmol/L (*Vitamin C*) + 100 µm H_2O_2	28.7±4.5	24.5±3.9	5.5±1.1	14.8±1.7	26.5±1.3	186.0±5.2
Control	97.5±1.5	1.5±0.5	0.5±0.5	0.5±0.5	0.0±0.0	4.0±3.0
93 µmol/L (*Vitamin C*) + 100 µm H_2O_2	29.8±3.7	19.5±3.2	7.2±1.1	15.2±2.6	28.3±2.3	192.7±3.4
Control	94.0±5.0	5.5±5.5	0.0±0.0	0.5±0.5	0.0±0.0	7.0±4.0
279.4 µmol/L (*Vitamin C*) +100 µmH_2O_2	34.3±3.4	15.3±3.3	7.7±1.4	16.7±2.4	26.0±4.9	184.7±12.1
Control	92.5±5.5	6.0±4.0	1.0±1.0	0.5±0.5	0.0±0.0	9.5±7.5

[a] Values represent duplicates from three experiment (means ± SEM). [b] Control samples with no H_2O_2

TABLE 3.1.1 The effect of *Rutin* treatment against oxidative DNA damage in human lymphocytes in the comet assay (SCGE assay)

Treatment	Percentage of the cells showing different grades of DNA damage[a]					
	No damage (<5%)	Low damage (5-25%)	Medium damage (25-45%)	High damage (45-70%)	Very High damage (>70%)	Total score (out of 400)
100 µmol/L H_2O_2 (*no Rutin*)	22.2 ±2.2	19.0± 3.7	9.7±2.1	22.2±3.6	27.0±.4.9	212.8±16.8
Control [b]	78.7±2.8	16.7±4.1	2.7±0.9	2.0±1.2	0.0±0.0	28.0±2.0
7.6 µmol/L (*Rutin*)+ 100 µm H_2O_2	21.3 ±1.9	16.7± 2.0	10.2±2.0	26.3±3.1	25.5±3.9	218.0±9.7
Control	81.7±.5.2	13.3±4.5	2.3 ± 1.9	2.3±0.9	0.3±0.3	26.3±7.6
23.3 µmol/L (*Rutin*) + 100 µm H_2O_2	22.7 ± 2.9	18.7± 3.2	11.7±2.0	25.5±2.3	21.5±1.9	205.0±8.2
Control	89±1.5	9.7±.1.5	1.0±0.0	0.3±0.3	0.0±0.0	12.7±1.9
93 µmol/L (*Rutin*)+ 100 µm H_2O_2	28.8 ± 4.1	22.7±3.8	12.3± 2.0	17.7±4.2	18.5± 2.0	173.7±13.8
Control	85.0± 5.1	11.7± 4.6	2.7±1.8	0.7±0.3	0.0±0.0	19.0±6.0
279.4 µmol/L (*Rutin*)+100 µmH_2O_2	33.2± 4.5	17.3±2.3	9.2±2.4	19.7±3.8	20.7±7.3	177.3±18.5
Control	84.3±6.7	7.3±4.8	6.7±2.4	1.0±0.0	0.7±0.7	26.3±9.1

[a] Values represent duplicates from three experiment (means ± SEM). [b] Control samples with no H_2O_2

Table 3.2. Comparison of the antioxidant effect of the flavonoids and vitamin C at the concentration of 279 μmol/L. Results are expressed as a percentage of the total DNA damage score obtained in the absence of the antioxidant (*mean* ± SEM, $n = 12$ for kaempferol, $n = 6$ for the other agents). Inter-agent comparisons were peformed by Tukey and Dunnett's tests as described in the text (*$p<0.05$, **$p<0.01$, ***$p<0.001$)

	Percentage of maximal DNA damage	Significantly more protective than:
Luteolin (L)	9 ± 2%	K*, Q_r***, A***, Q3g***,VitC***, R***
Myricetin (M)	10 ± 3%	K*, Q_r***, A***, Q3g***, VitC***, R***
Quercetin (Q)	22 ± 3%	A***, Q3g***, VitC***, R***
Kaempferol (K)	32 ± 3%	A**, Q3g**, VitC***, R***
Quercitrin (Q_r)	45 ± 9%	VitC***, R***
Apigenin (A)	59 ± 2%	R*
Quercetin-3-glucoside (Q3g)	62 ± 5%	
Vitamin C (VitC)	78 ± 5%	
Rutin (R)	83 ± 9%	

Tables 3.3 The rankings of antioxidant activity of flavonoids tested in the present study and some vitamins in order of decreasing potency using different methods

Ranking of antioxidant activity in order of decreasing potency	Methods	References
Quercetin, myricetin, rutin, trolox, vitamin C, kaempferol, α-tocopherol, ß-carotene, apigenin	lipoprotein oxidation model	(Vinson J et al, 1995)
Myricetin, quercetin	antioxidant against peroxide (induction period of lard at 60° C)	(Mehta B et al, 1958)
Quercetin, luteolin, quercitrin	stability of lard at 100°C	(Hudson B et al, 1983)
Quercetin, myricetin, kaempferol, apigenin	lipid peroxidation in red blood cells membrane	(Chen Z et al, 1996)
Quercetin, myricetin, rutin, quercitrin	xanthine-xantine oxidase system	(Robak J et al, 1988a)
Myricetin, quercetin, rutin, quercitrin	superoxide generation by Fenton methosulphate model	(Robak J et al, 1988a)
Myricetin, quercetin, rhamnetin, apigenin, kaempferol	hydroxyl radical scavenging activity	(Husain S et al, 1987)
luteolin, quercetin, kaempferol, apigenin	nonenzymic lipid peroxidation in rat liver	(Galvez J et al, 1995)
luteolin, kaempferol, rutin, quercetin, myricetin	Fenton reagent assay (Fe^{2+}/H_2O_2)	(Shimoi K et al, 1994)
Kaempferol, luteolin = rutin, quercetin	antioxidation of linoleic acid inhibition	(Torel J et al, 1986)
Quercetin, kaempferol, rutin	free radical scavenging mechanism in meat	(Shahidi F et al, 1992)
luteolin, apigenin	CCl_4 induced microsomal lipid peroxidation	(Cholbi M et al, 1991)
Myricetin, α-tocopherol, ß-carotene	inhibition of strand breaks in plasmid by singlet molecular oxygen	(Davasagayam T et al, 1995)
Quercetin, α-tocopherol	inhibition of human low-density lipoprotein model	(Frankel E et al, 1993)
Myricetin, quercetin, quercitrin, rutin	lipid peroxidation in corn oil	(Pratt D et al, 1990)
Quercetin, luteolin, rutin	lipid peroxidation in lard	(Pratt D et al, 1990)
Quercetin, rutin	autooxidation of rat cerebral membranes assay	(Saija A et al, 1995)
Quercetin, luteolin, myricetin, kaempferol, quercitrin, quercetin-3-glucoside, apigenin, rutin, vitamin C (ED50 in the comet assay on human lymphocytes DNA)		(Noroozi M et al, 1998a)

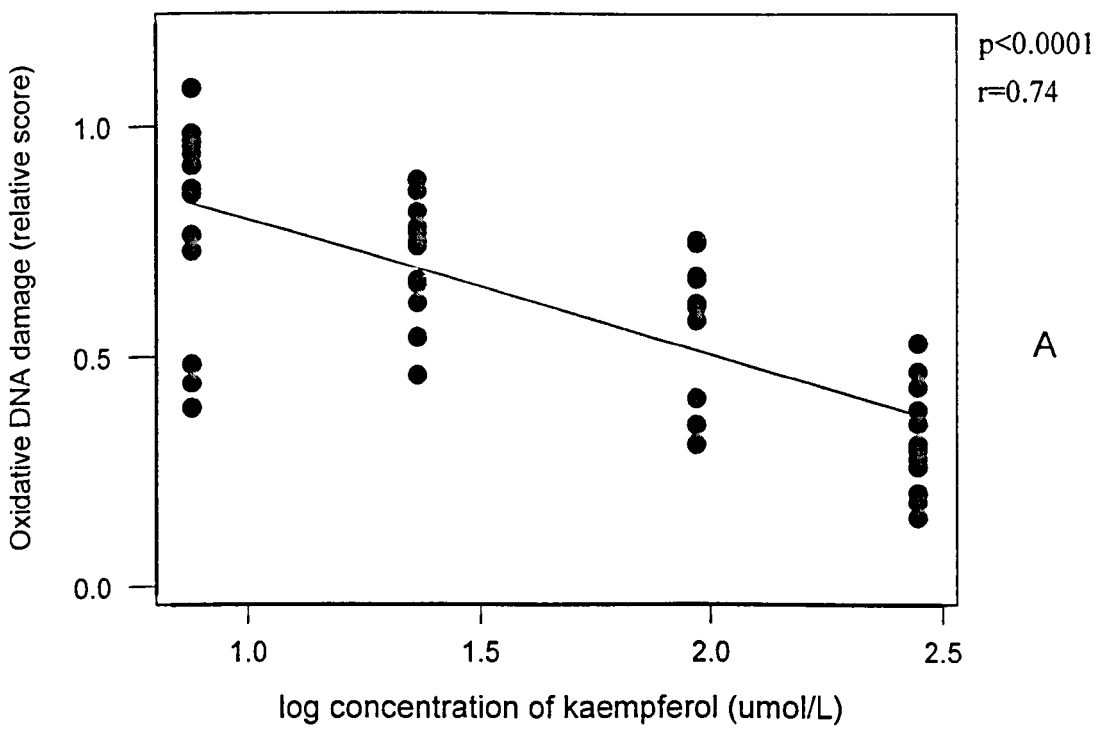
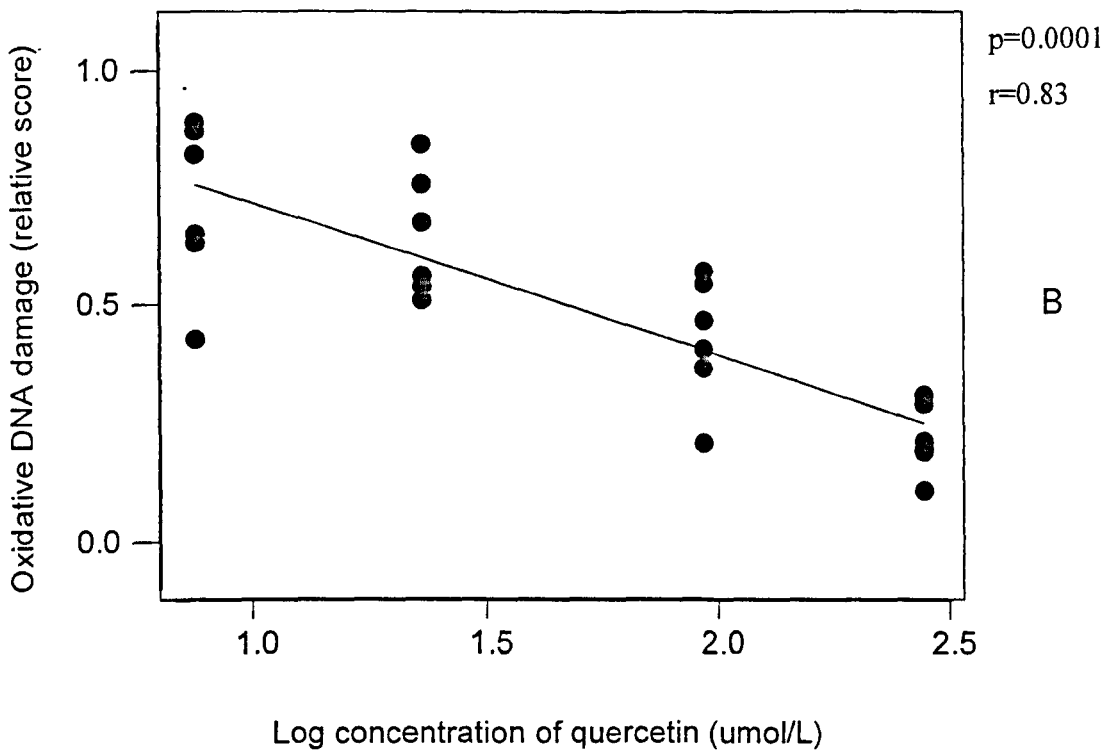
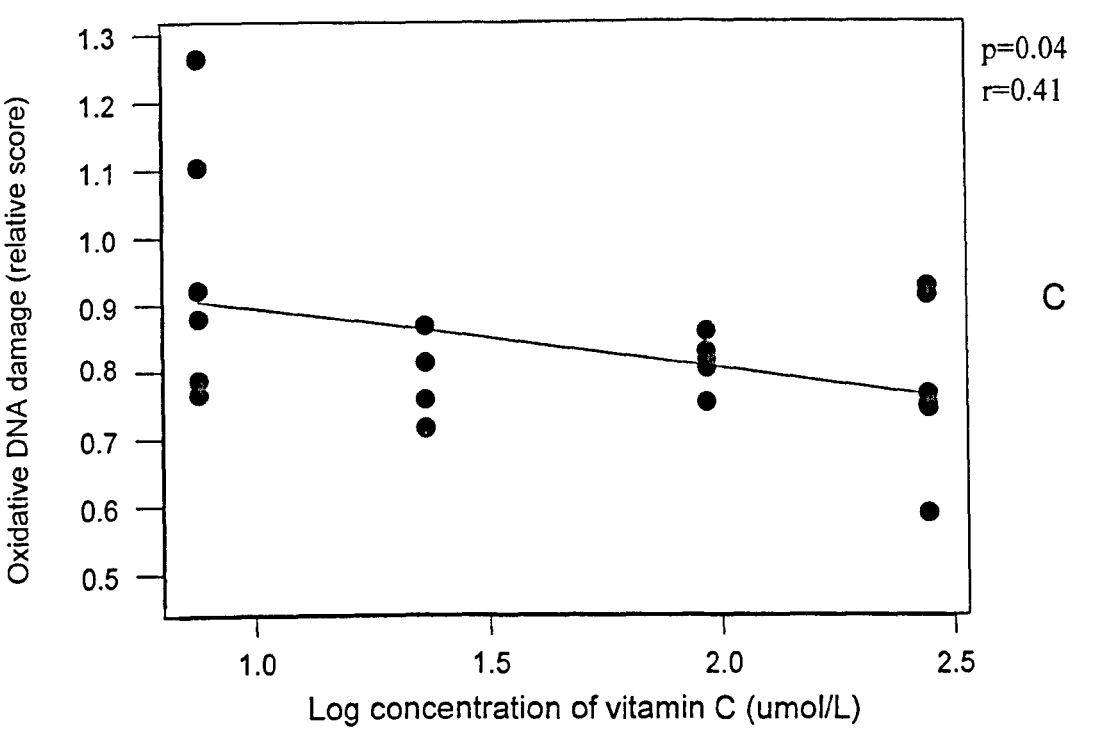


FIGURE 3.1. The antioxidant effect of kaempferol (A), quercetin (B) and vitamin C (C) against oxygen radical -generated oxidative DNA damage in human lymphocytes in the comet assay. There are statistically significant dose response ralationships with significant ($p< 0.0001$), (vitamin C, $p=0.04$) protection effects for each coumpound.


p=0.0001
r=0.83
B
Oxidative DNA damage (relative score)
1.0
0.5
0.0
1.0
1.5
2.0
2.5
Log concentration of quercetin (umol/L)


p=0.04
r=0.41
C
Oxidative DNA damage (relative score)
1.3
1.2
1.1
1.0
0.9
0.8
0.7
0.6
0.5
1.0
1.5
2.0
2.5
Log concentration of vitamin C (umol/L)

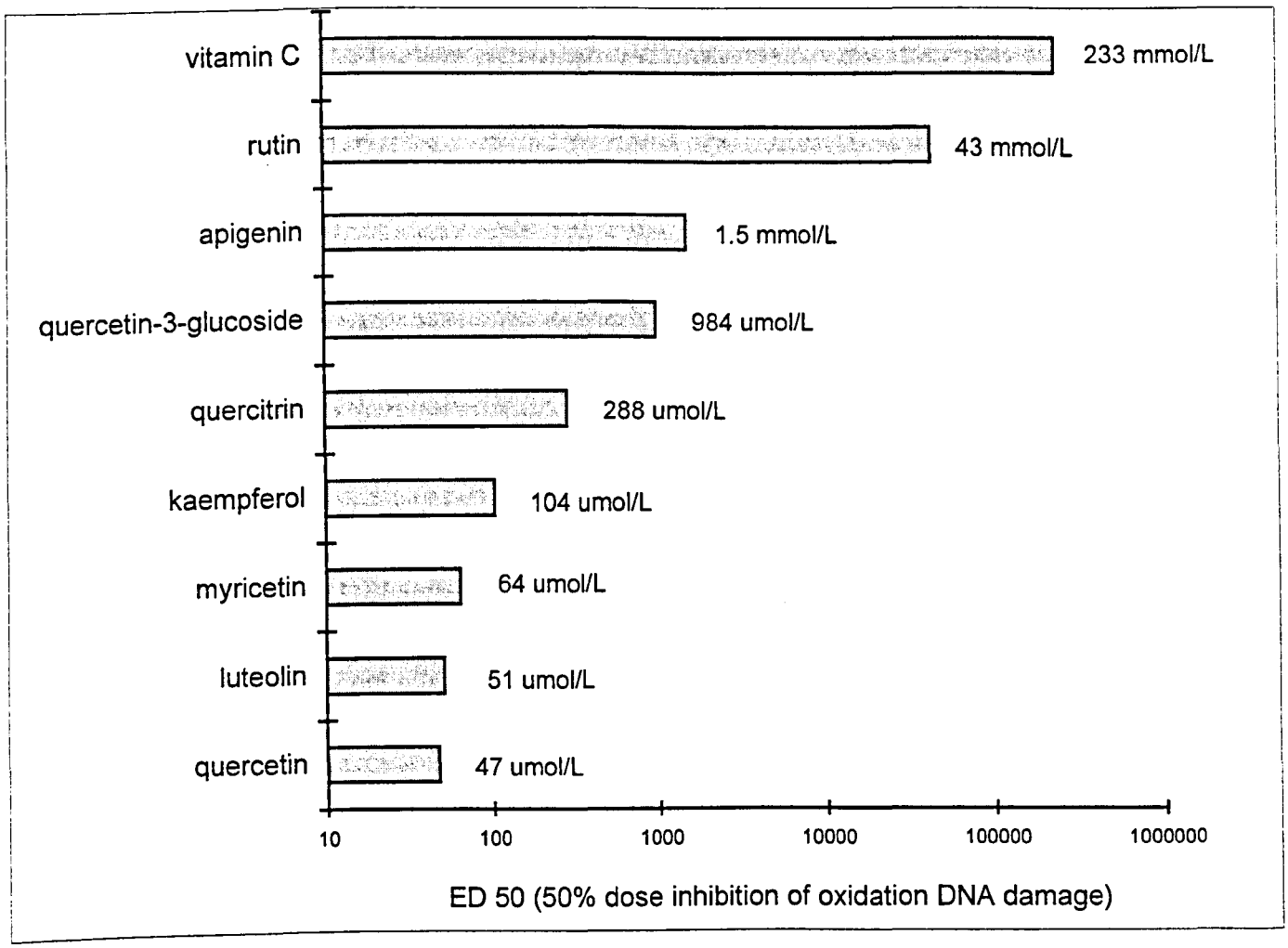


FIGURE 3.2. Comparison of the total antioxidant activities of flavonoids on human lymphocytes in the comet assay assessed by estimated dose which would result in 50% reduction in oxidative DNA damage from unopposed H_2O_2 (100μmol/L).

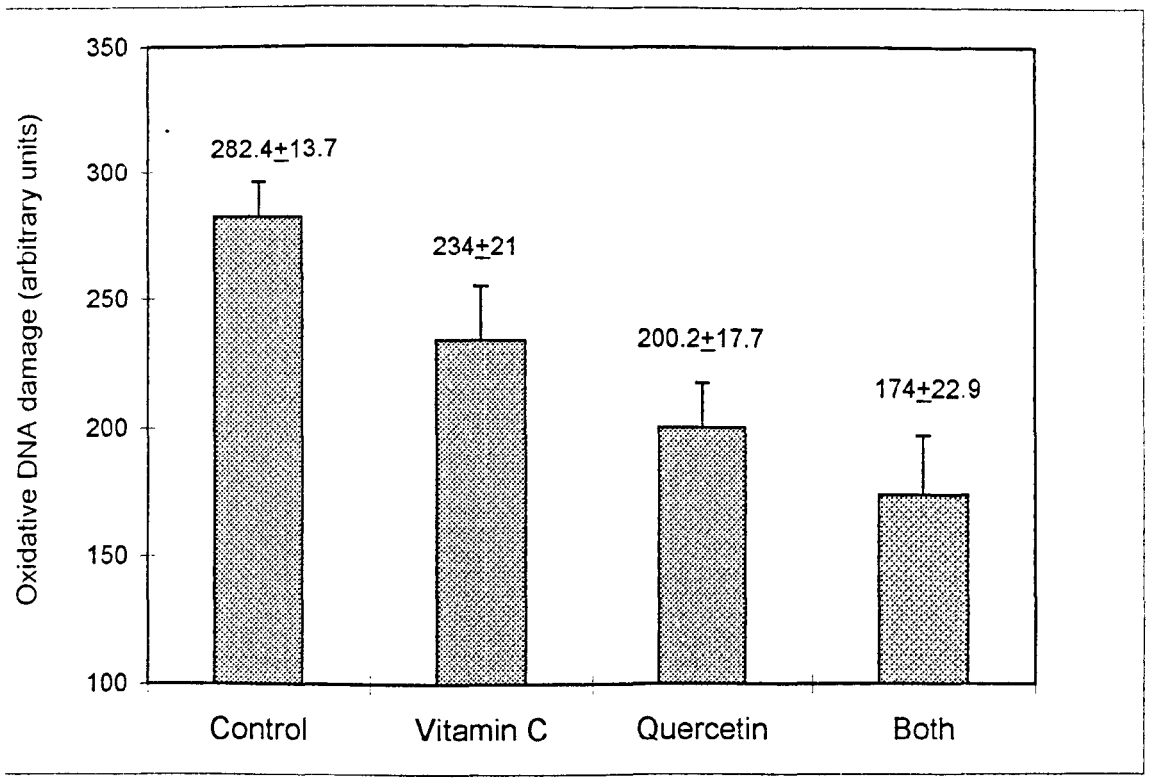


FIGURE 3.3. Antioxidant activities of flavonoids (quercetin) and L-ascorbic acid (vitamin C) each 23μmol/L on human lymphocytes in the comet assay. Values represent *means* (±SEM) from 500 cells pretreated with each substance. Quercetin was significantly more protective than vitamin C against oxygen radical -generated oxidative DNA damage (Quercetin:$p< 0.0001$), (Vitamin C: $p=0.03$).The effect of quercetin and vitamin C were additive when both were at the same concentration.

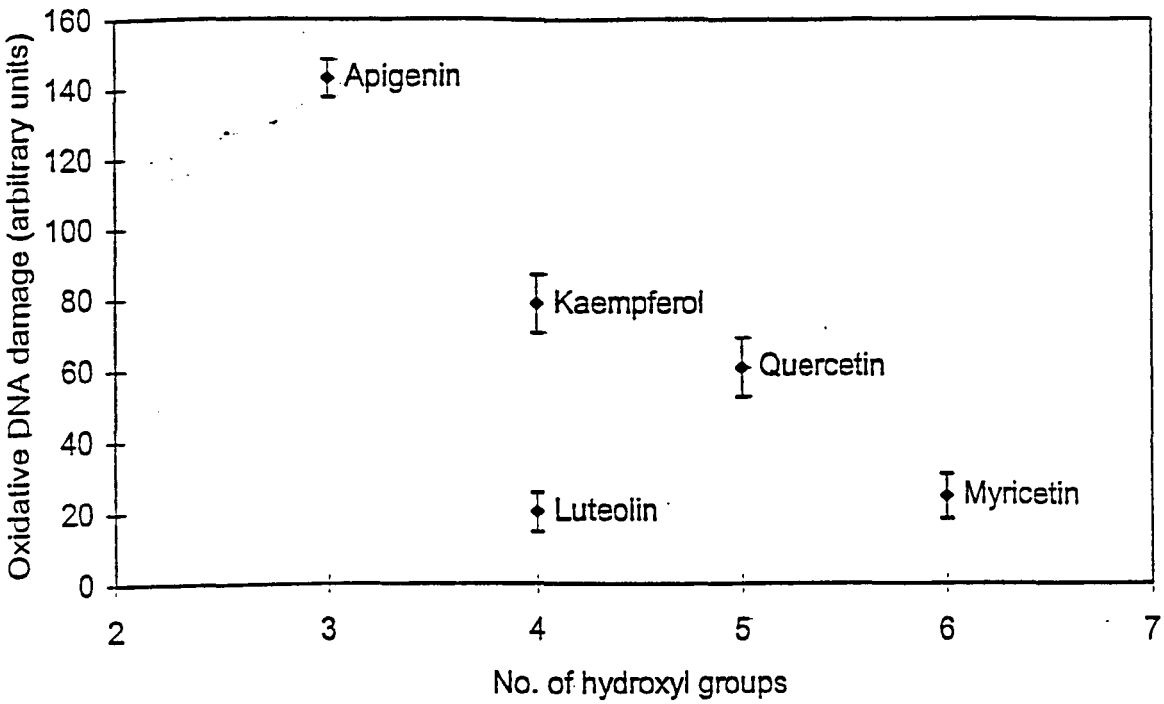


FIGURE 3.4. The effect of free flavonoids on oxidative DNA damage on human lymphocytes in the SCGE assay, plotted against the number of hydroxyl groups in the flavonoids. Data show *mean* ±SEM, n=6-12 samples, at the concentration of 279.4 μmol/L.

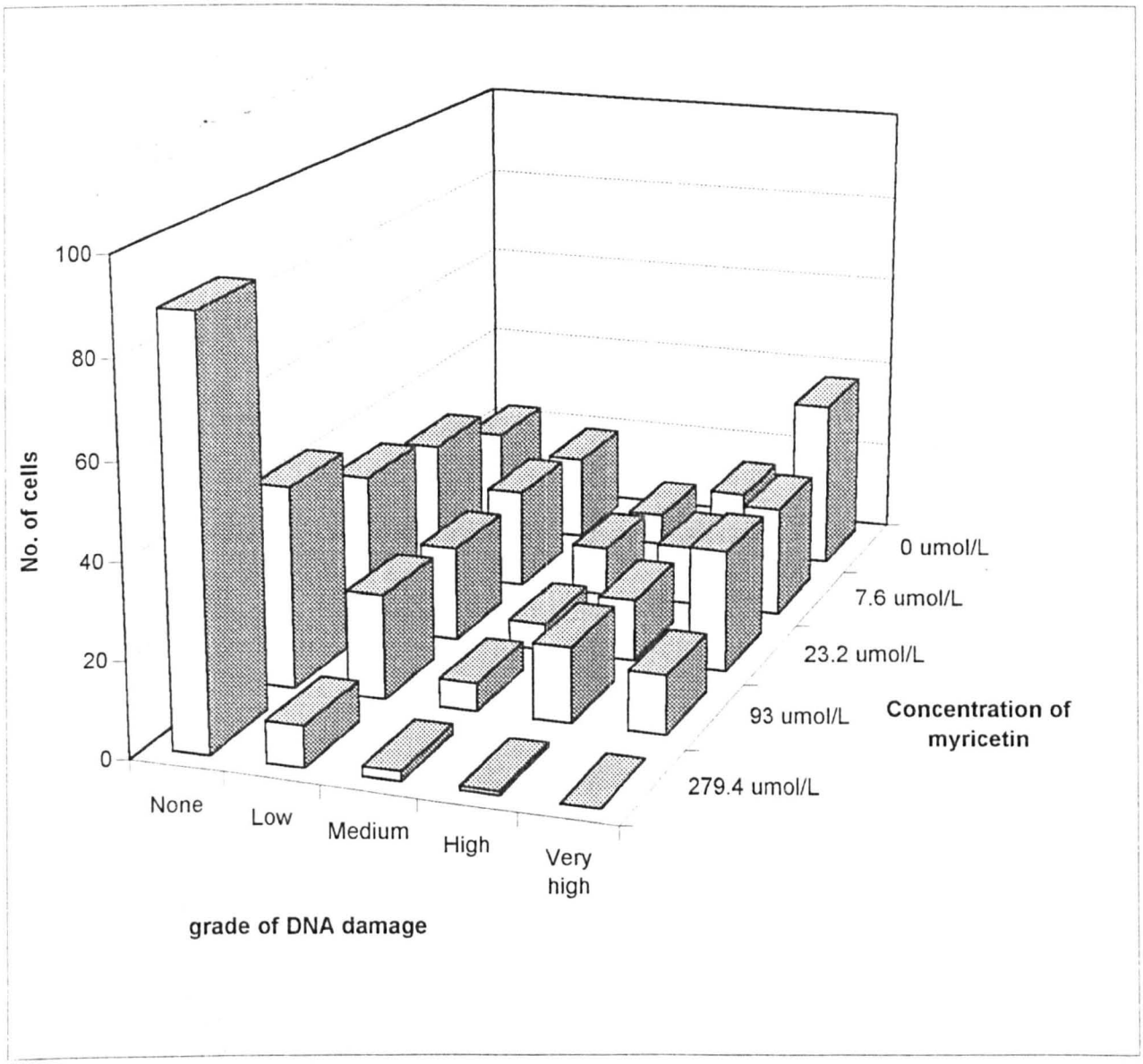


FIGURE 3.5. The antioxidant effect of myricetin against oxygen radical -generated oxidative DNA damage in human lymphocytes in the comet assay. There is a statistically significant dose response relationship with significant ($p< 0.0001$) protection against effects of oxygen radical.

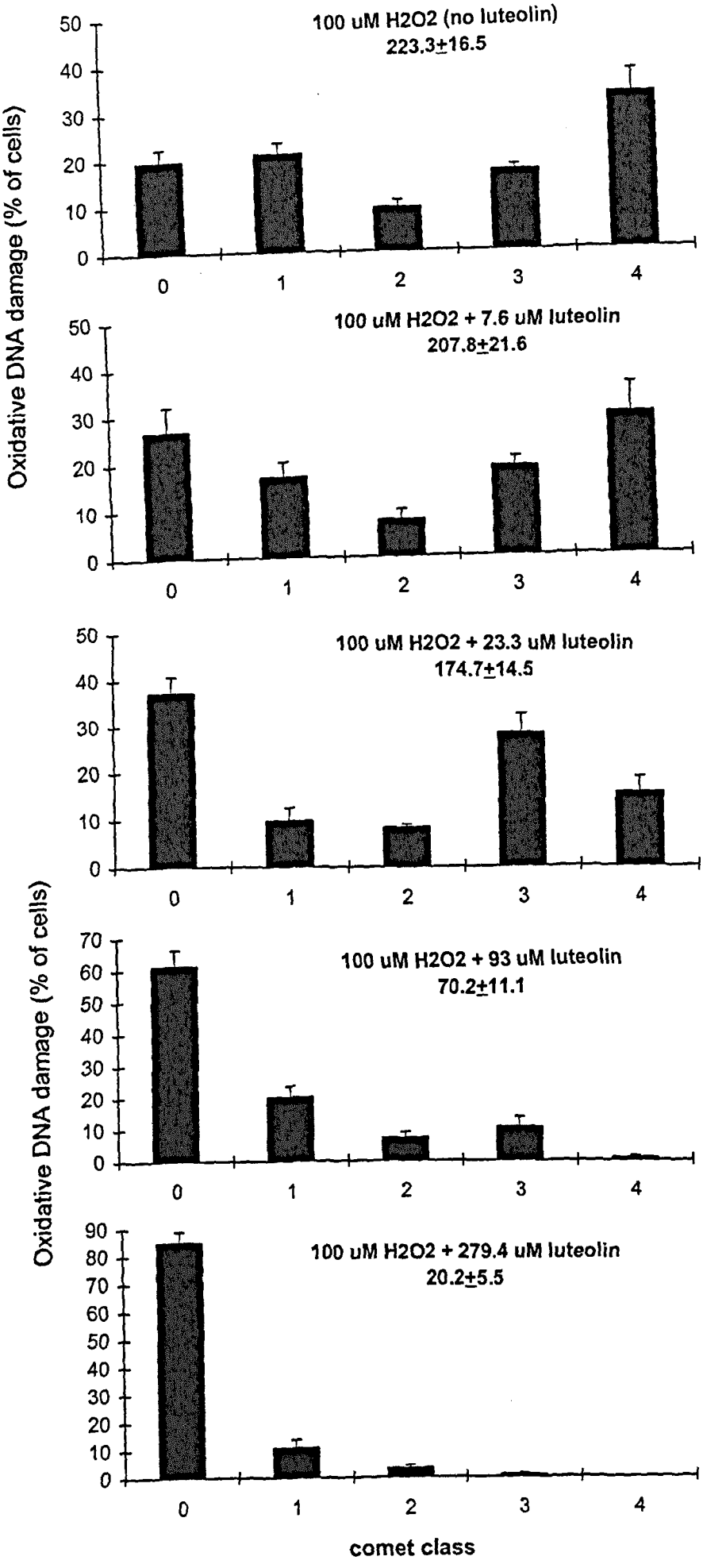


FIGURE 3.6. Example of results. Hydrogen peroxide-induced DNA damage in human lymphocytes, with or without luteolin pretreatment, is expressed as the frequency of comet classes amongst 100 cells counted at each luteolin concentration using SCGE. Results are mean±SEM for n≥ 6.

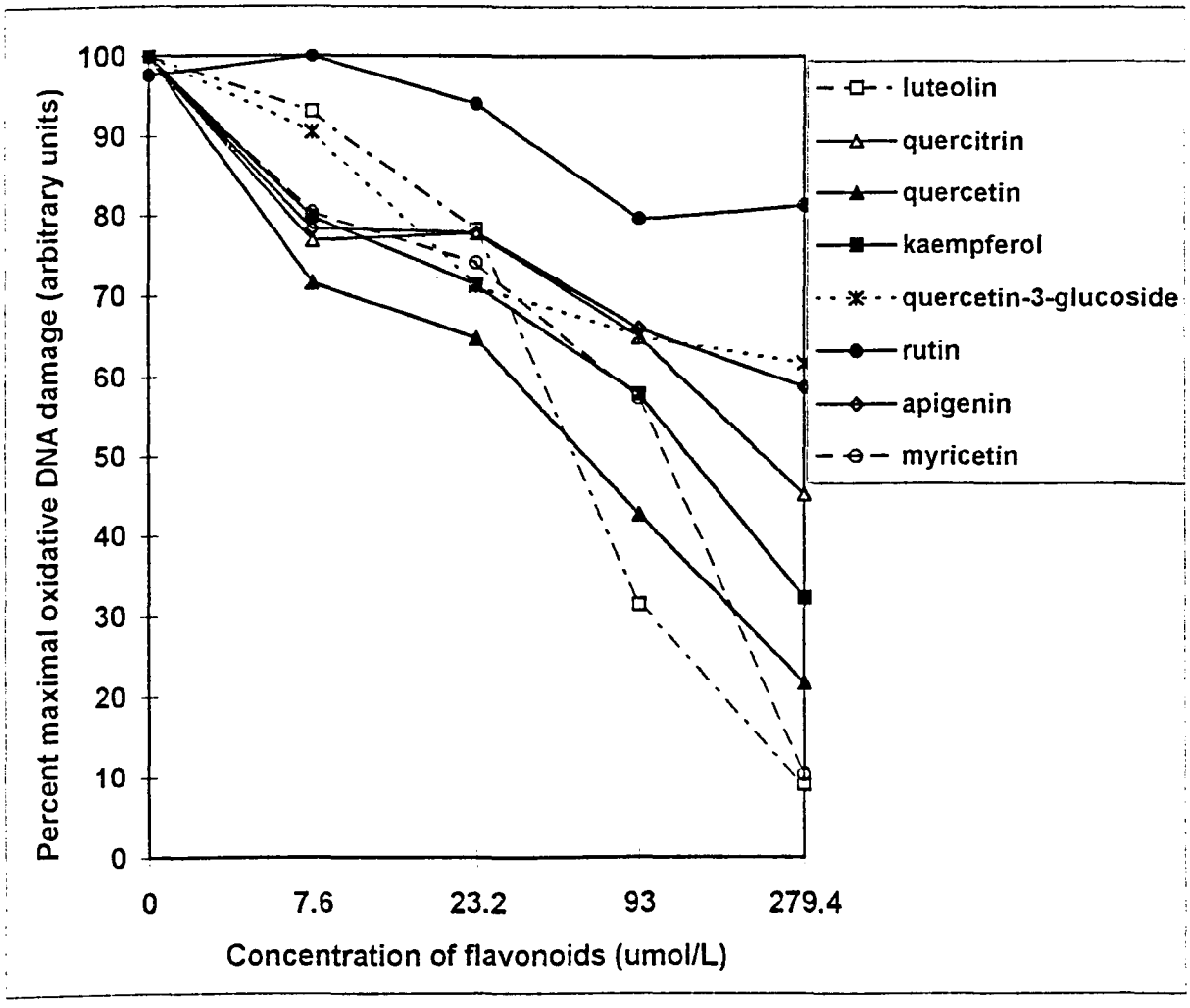


FIGURE 3.7. The antioxidant activity of flavonoids against oxygen radical -generated oxidative DNA damage in human lymphocytes in the comet assay (SCGE assay).

CHAPTER 4: Total antioxidant activity of vitamin C and flavonoids

(paper submitted to American Journal of Clinical Nutrition, co-authors H Miller, N Sattar, MEJ Lean)

Abstract

To compare the antioxidant activities of some common flavonoids with vitamin C and to evaluate the effect of in vitro addition of flavonoids on the total antioxidant activity of human plasma. The total antioxidant activities of 17 free and conjugated flavonoids and related polyphenolic compounds at the concentrations of 1 mmol/l were tested in vitro and compared with vitamin C at the same concentrations. The total antioxidant activity of human plasma was measured before and after adding rutin, quercetin and 100 μmol/l kaempferol in concentration 10-100 μmol/l.

All flavonoids tested except naringin had more antioxidant activity than vitamin C ($p<0.05$). Quercetin and rutin produced dose-related increases in antioxidant capacity of human plasma. The addition of 50 μmol/l quercetin and 100 μmol/l quercetin, rutin and kaempferol significantly increased the total antioxidant capacity of human plasma ($p<0.001$). There was strong positive correlation between the number of hydroxyl group of flavonoids and the antioxidant activity ($p<0.001$, $r = 0.85$). The flavonoid aglycones were more potent in their anti-free

radical action than their corresponding glycosides ($p<0.05$). This evidence indicates a potent antioxidant action of dietary flavonoids, of potential importance in protection against cardiovascular disease and cancer.

4.1 Introduction

The aim of this study was to determine the antioxidant capacities of various dietary flavonoids compared to vitamin C, and their antioxidant potential in human plasma. Antioxidant rich nutrients provide part of the defence mechanism in conjunction with endogenous antioxidants such as uric acid. The importance of dietary antioxidants in the maintenance of health and protection from disease is becoming increasingly well recognised (Miller et al 1995).

Vitamin C (ascorbic acid), α-tocopherol, and β-carotene are well established antioxidants (Serafini et al 1996) whose importance may have been distorted since they are relatively easily and widely measured, but occur in foods which also contain other less familiar antioxidant compounds. Flavonoids are being added to the list of potentially significant diet-derived antioxidants.

Table 4.1 shows a range of some antioxidants in the body, and **table 3.3** shows the rankings of antioxidant potency, using a range of methods and including the present thesis. For the present study we have employed the in vitro Trolox equivalent antioxidant capacity (TEAC) assay to rank the potency of vitamin C

and 17 flavonoids and polyphenols chosen either because they are common in foods or to provide a range of structures. Also we adapted the standard technique to examine the influence of flavonoids on the antioxidant capacity of human plasma.

Material and Method

Study design and methods are explained in detail in Chapter 2, part 2.4

Statistical methods

Explained in Chapter 2, part 2.8.2

4.2 Results

4.2.1 Total antioxidant activities of vitamin C and flavonoids

Solutions of 17 flavonoids, polyphenoles and vitamin C were tested in the TEAC assay. The solvents used were ethanol except hesperidine, for which pyridine was used. Absolute ethanol had negligible antioxidant activity (0.07 mmol/l) or no antioxidant activity in previous study (Miller et al 1993). Pure pyridine solution used had TEAC 0.94 mmol/l. This value was subtracted from the results of hesperidin in solution, since the concentration of pure pyridine remained relatively unaltered, when hespiridin was added.

The results of the TEAC assay showed all flavonoids and vitamin C have antioxidant activity. Because of the high potency of many of the compounds total exceeding that of Trolox several-fold, the standard TEAC sample concentration of 1 mmol/l was reduced to 300 μmol/l. Flavonoids at 1 mmol/l had antioxidant activities between range of 0.83 – 6.49 mmol/l Trolox equivalents. In order to relate the results of the present study to TEAC data in the literature (**Table 4.2, Figure 4.3, Figure 4.3**). All flavonoids and polyphenols (except naringin) have greater antioxidant potency than vitamin C on a molar basis ($p<0.05$) (**Table 4.3**). A summary of literature reports of relative antioxidant activities of flavonoids is given in **Table 3.3**.

4.2.2 The effect of chemical structure of flavonoids on antioxidant activity

The characteristics of flavonoids tested in present study ranged from epigallocatechin gallate (EGCG) with 8 hydroxyl groups to chrysin with 2 hydroxyl groups. Distribution of the number of hydroxyl groups were: epigallocatechin gallate (8-OH); epicatechin gallate (ECG) (7-OH); myricetin (6-OH); quercetin and catechin (5-OH); isorehamnitin, quercitrin, quercetin-3-glucoside (Q-3-g), rutin, luteolin and kaempferol (4-OH); naringenin, apigenin, silymarin, (3-OH); naringin, hesperidin and chrysin (2-OH) (**Figure 2.4**).

Higher numbers of hydroxyl groups in ring A, B or C of flavonoids and polyphenols significantly increased the TEAC ($p<0.001$, $R = 0.85$) (**Figure 4.1**) There was a positive relation between the number of hydroxyl groups of

flavonoids and total antioxidant activity of flavonoids. The highest TEAC was for epicatechin gallate, with the 7 hydroxyl groups (6.49 mmol/l) (**Table 4.2**) and the lowest TEAC was in naringin with 2 hydroxyl groups was (0.83 mmol/l).

The number and the position of hydroxyl groups in ring A, B and C of flavonoids (**Figure 2.4**) also glycosylation of flavonoids may have an effect on antioxidant activity. The relation between chemical structure of flavonoids used in present study and TEAC merits consideration. Quercetin and catechin both have 5 hydroxyl groups but TEAC of quercetin is signficantly more than catechin (4.67 $\pm$ 0.45) versus (3.10 $\pm$ 0.09), which may be attributable to the 2,3 double bond and 4-oxo group in C ring of quercetin (**Figure 2.4, Table 4.2**).

4.2.3. Influence of glycosylation on antioxidant activity

Glycosylation of quercetin (at 3-hydroxyl group in the C ring) to form rutin (3-rutinoside), quercitrin (3-L-rhamnoside) and quercetin-3-glucoside decreases the antioxidant activity of these substances. Similarly methylation at 3-hydroxyl groups of ring C in isorhamnetin reduces the antioxidant activity.

Total antioxidant activity of quercetin (4.67 $\pm$ 0.45) is significantly greater than rutin (3.18 $\pm$ 0.12), quercitrin (3.35 $\pm$ 0.02) and quercetin-3-glucoside (3.65 $\pm$ 0.12) as glycosylated form of quercetin (**Table 4.2, Figure 2.4**). Glycosylation of naringenin to form naringen (7-rhamnoglucoside) gave a 60% reduction in antioxidant activity (2.53 $\pm$ 0.09) versus (0.83 $\pm$ 0.00) ($p<0.05$). The result of

our previous study indicated that the aglycones quercetin, luteolin, myricetin and keampferol have a more protection against oxygen radical on human lymphocytes than glycosylate flavonoids (Noroozi et al 1997 & 1998a). This is in agreement with the results of several other studies using a wide range of methods for assessing antioxidant capacity (**Table 3.3**).

4.2.4 Total antioxidant capacity of flavonoids added to fresh human plasma

To test the effect of adding flavonoids to fresh human plasma we chose quercetin (an aglycone) and quercetin-3-rutinoside (rutin, a conjugated quercetin) and kaempferol (aglycone). Total antioxidant capacity of fresh heparinised plasma in this study was 1.394 ± 0.043, ($n = 10$).

Significant increases were measured when 50 or 100 µmol/l quercetin and 100 µmol/l rutin and kaempferol were added to the plasma. Linear correlation suggested a dose-related effect from 10 to 100 µmol/l for quercetin ($R2 = 98.9\%$, $p<0.001$) and for rutin ($R2=95.3\%$, $p=0.004$) (**Table 4.4**).

4.3 Discussion

In our previous study quercetin was the most protective flavonoid against oxygen-radical generated DNA damage to human lymphocytes in the comet

assay (Noroozi et al 1997 and 1998a). The present study using TEAC extends our understanding of these compounds, which are more potent antioxidants than vitamin C or vitamin E (Trolox). Our work (Comet assay) showed quercetin and myricetin, with hydroxyl groups at the positions 3', 4' containing the unsaturated 2, 3-double bond in the C ring, were the strong antioxidants (**Figure 2.4, Table 3.3**). This agrees with the work of other researchers, (Chen et al 1996; Shahidi et al 1992) which relates the position and number of hydroxyl groups to antioxidant capacity. The total number of hydroxyl groups is considered a crude indicator of antioxidant capacity. For maximal radical scavenging, it appears that the 3-OH should be associated with a group 2, 3-double bond and 4-carbonyl in the C ring . The availability of phenolic hydrogens as hydrogen donating radical scavengers is believed to explain their antioxidant activity (Rice-Evans et al 1996). Polyphenols and flavonoids with a 3', 4' dihydroxy configuration such as quercetin are believed to possess particularly strong antioxidant activity (Shahidi et al 1992) (**Figure 2.4**).

The present study provides a ranking of antioxidant activities similar to previous studies and supporting the view that the number of hydroxyl groups and the conjugation status are both important in determining antioxidant activity. Glycosylation of flavonoids consistently reduced the TEAC compared with aglycone flavonoids. These results are supported by other rankings of antioxidant activity using different methodologies. Both rutin and quercitrin (glycated compounds) show lower antioxidant activity than aglycone quercetin

(Robak et al 1988a; Ratty et al 1988; Pratt et al 1990). These findings are of pratical relevance since most flavonoids in foods occur as conjugates, not as aglycones (Crozier et al 1997a).

The adaptation of the TEAC assay to test the putative antioxidants in fresh human plasma in supra-physiological concentrations (10, 20, 50, 100 μmol/l), produced results which could support a physiological role for flavonoids in the body's antioxidant defence system (**Table 4.4**). As before, quercetin proved the most potent, above kaempferol and rutin at 100 μmol/l. Increasing concentrations of both quercetin and rutin from 10 to 100 μm resulted in dose-related responses with $R^2 > 90\%$ from linear regression.

Paganga et al (1997) showed the concentrations of polyphenols in plasma is 0.5 - 1.6 μmol/l and this level may have important effects on antioxidant capacity of plasma. The TEAC assay results were suggestive of a detectable effect of quercetin at 10 μmol/l in plasma, although in this study the effect was not clearly statistically significant until 50 μmol/l.

The physiological importance of flavonoids depends on their antioxidant activities but also on their availability from the diet and the extent to which they may be dehydrosylated. The analysis of flavonoids in human diet, plasma and urine is now possible (Crozier et al 1997a; Paganga et al 1997;Gross et al 1996; Ameer et al 1996; Hollman et al 1995) and the factors which influence

absorption of free and conjugate form of flavonoids by human gut are becoming better understood. The absorption rate for quercetin aglycone was found to be very variable with mean of 24% but the absorption from onions was 52%, which suggests that quercetin glucosides of onions are better absorbed than the aglycone (Hollman et al 1995).

The mechanisms and the sites of absorption of flavonoids in humans and their bioavailability in general have not yet been elucidated . Rutin and quercetin, glycosides are absorbed by human gut (Paganga et al 1997) and the time course for absorption of quercetin from onions corresponds with our own unpublished findings.

The results of Knekt et al (1996) and Hertog et al (1993a) suggest that people with very low intakes of flavonoids have higher risks of coronary heart disease. The habitual intake of flavonoids (flavonol) and of their major food source, tea, may also protect against stroke (Keli et al 1996). Drinking of tea increases the antioxidant capacity of plasma (Serafini et al 1996). Hertog et al (1997) considered tea to be the most important source of dietary flavonol for people who do not drink red wine. Tea is a major source of dietary polyphenols and rich source of flavonols, catechins and catechin esters (Shahidi et al 1992; Rice-Evans et al 1995). Our results on the TEAC of catechin, catechin esters (ECG and EGCG) and rutin indicate the antioxidant properties of these compounds which are found in teas. ECG had the greatest antioxidant activity of the 17

flavonoids and polyphenols tested, seven fold greater than vitamin C. In the TEAC assay all these flavonols were antioxidants (**Table 4.2**) and in decreasing order of potency are: ECG $\simeq$ EGCG > rutin > catechin. The antioxidant activity of catechin esters (gallic acid linked to catechin) was also supported by Guo et al (1996) and Salah et al (1995).

As well as tea and many fruits and vegetables contain flavonols and flavone glycosides in high concentrations (Herrman et al 1988, Crozier et al 1997a). Estimating the overall impact of dietary flavonoids on health will depend on understanding the antioxidant activity of the specific compounds and their conjugates - demonstrated in the present study. We also need to know that foods with absorbable flavonoids are being consumed, and how the food is prepared since storage and cooking may effect composition (Crozier et al 1997a). When this detailed information is available, a better indication of the health impact of flavonoids will be possible than can be derived from simplistic application of "food tables" of the total aglycone flavonoid contents.

From the results of the present study, the flavonoids most likely to be of relevance to human diets and health are quercetin-3-glucoside, quercitrin and rutin, as the most potent flavonoid conjugates, which are likely to be absorbed. The concentrations of flavonoids detected in human plasma (**Table 4.1**) might be too low to have a major impact on the antioxidant capacity of plasma, but it is possible that these compounds may concentrate in tissues.

Table 4.1 - Antioxidant defences in human plasma and some polyphenolic and flavonoid antioxidants detected in human plasma and urine

Antioxidant proteins	Small molecule antioxidants	Flavonoid and polyphenolic antioxidants[2]	Antioxidant flavonoids detected in human plasma and urine
	Lipid-soluble (lipoprotein-associated)[1]:		Plasma
Enzyme[1]:		EPCG[+]	quercetin (24% absorption of orally administration)[3]
Catalase	α-tocopherol (15-40 μM)	EPGCG[++]	quercetin glucoside[3]
Cu, Zn superoxide dismatase (Mitocondria)	Ubiquinol-10 (0.4-1.0 μM)	Quercetin	diosmin (50-400 ng/ml)[6]
(5-20 units/ml)	Lycopene (0.5 - 1.0 μM)	Myricetin	quercetin (196 ng/ml)[6]
Glutation peroxidase (GSH)(0.4 units /ml)	β-carotene (0.3 - 0.6 μM)	Isorhamnetin	3-o-methyl-catechin (11-18 μg/ml)[6]
Ceruloplasmin (ferroxidase activity)	Lutein (0.1 - 0.3 μM)	Quercetin-3-glucoside	rutin (0.76-0.72 μM)[6]
(0.18 - 0.40 g/l)		Chrysin	quercetin rutinoside[3]
		Quercitrin	quercetin glycoside (0.60-1.34 μM)[6]
		Rutin	phloridzin (dihydrochalcone) (0.60-1.64 μM)[6]
Non Enzyme:[1]	Water Soluble:[1]	Luteolin	Quercetin (<1%)[7]
Albumin (38-52 g/l)	Ascorbic acid (30-150 μM)	Kaempferol	Naringin and hesperidin[9,8]
Lactoferrin (0.03-0.28 mg/l)	Glutathione (<2 μM)	Silymarin	
(Ferritin) (0.2-0.44 mg/l)	Uric acid (160-450 μM)	Catechin	
Protein thiols (350-500 μM)	Bilirubin (5-20 μM)	Naringenin	
Transferrin (1.5-3.4 g/l)	D-mannitol[4]	Hesperidin	Urine
Ceruloplasmin (0.18-0.40 g/l)		Naringin	quercetin metabolite:
Haptoglobin (0.5-3.6 g/l)		Apigenin	(homoprotocatechuic acid) (0.7 μg/ml)[5]
Hemopexin (0.5-1.2 g/l)			(homovanillic acid) (2.8 μg/ml)[5]
N-acetyl cysteine ferritin (0.02-0.44 mg/l)			(metahydroxy phenylacetic acid) (4.8 μg/ml)[5]
			Naringin and hesperidin (<25%)[9,8]

1 Frei B et al (1992)
2 Noroozi M et al (present study)
3 Hollman PCH et al (1995)
4 Jeng J et al (1994)
5 Gross M et al (1996)
6 Paganga G et al (1997)
7 Gugler(1975)
8 Booth AN et al (1958a,b)
9 Ameer B et al (1996)
\+ epicatechin gallate
++ epigallocatechin gallate

Table 4.2 Characteristics of flavonoids, polyphenols and Vitamin C with antioxidant capacity (1 mmol/L Trolox equivalent)

Compound	Family	Free hydroxyl positions	Conjugation	TEAC (mmol/L) (mean ± SD)	Major food and plant source
Epicatechin gallate	4	5,7,3',4',3",4",5"	Aglycone	6.49 ± 0.000	green and black tea
Epigallocatechin gallate	4	5,7,3',4',5',3",4",5"	Aglycone	5.99 ± 0.094	green and black tea
Quercetin	1	3,5,7,3',4'	Aglycone	4.67 ± 0.045[4]	Onions, lettuce, apple skin, berries brocolli
Myricetin	1	3,5,7,3',4',5'	Aglycone	4.53 ± 0.13[4]	grapes, cranberry
Isorhamnetin	1	3,5,7,4'	3'-OCH3	4.43 ± 0.52	onions, plant foods
Quercetin-3-glucoside	1	5,7,3',4'	3-glucoside	3.65 ± 0.12	onions, plant foods
Chrysin	2	5,7	Aglycone	3.45 ± 0.07	fruit skins**
Quercitrin	1	5,7,3',4'	3-L-rhamnoside	3.35 ± 0.02	plant foods***
Rutin	1	5,7,3',4'	3-rutinoside	3.18 ± 0.12	Tea
Luteolin	2	5,7,3',4'	Aglycone	3.16 ± 0.09	Celery
Silymarin	1	5,7,4' *	3' – OMe	3.15 ± 0.02	Fruit of silybum marianum (milk thistle used in folk medicine)
Catechin	4	3,5,7,3',4'	Aglycone	3.10 ± 0.09	Tea
Apigenin	2	5,7,4'	Aglycone	2.96 ± 0.09[5]	celery, parsley
Naringenin	3	5,7,4'	Aglycone	2.53 ± 0.09	Citrus fruits (orange, lemon, grapefruit)
Kaempferol	1	3,5,7,4'	Aglycone	2.49 ± 0.13[4]	Leek, radish, endive, brocolli, tea, grapefruit
Hesperidin	3	5,3'	7-rhamnoglucoside	2.40 ± 0.14	[++]citrus fruits (grapefruit, sour orange juice)
Naringin	3	4',5	7-rhamnoglucoside	0.83 ± 0.00	Grapefruit, orange
Vitamin C	--	--	--	0.91 ± 0.22[6]	fruits and vegetables

Family: (1 = Flavonol) (2 = Flavone) (3 = Flavanone) (4 = Flavanol)

* 5,7,4' - Trihydroxy-3'-methoxydihydro flavonol (Harborne et al 1975) and flavolignan (Vinson et al 1995)

** Sedative folk medicine and plants e.g. Chrysanthemum morifolium (Hu et al 1994)

[++](Mouly et al 1993)

*** e.g. hypericum brasiliense (Rocha et al 1995)

All experiments were conducted in duplicate except [4](n=6), [5](n=3) and [6](n=10)

Table 4.3 Comparison of the antioxidant effect of the flavonoids, polyphenol and vitamin C. The flavonoids are listed in decreasing order of potency.

	EPCG[1]	EPGCG[2]	Quercet	Myri	Isor	Q-3-G[3]	Chry	Quercit	Rutin	Lute	Sily	Cate	Api	Nar	Kae	Hes	Vit. C
EPGCG	NS																
Quercetin	*	*															
Myricetin	*	*	NS														
Isorhamnetin	*	*	NS	NS													
Quercetin-3-G	*	*	*	*	NS												
Chrysin	*	*	*	*	*	NS											
Quercitrin	*	*	*	*	*	NS	NS										
Rutin	*	*	*	*	*	NS	NS	NS									
Luteolin	*	*	*	*	*	NS	NS	NS	NS								
Silymarin	*	*	*	*	*	NS	NS	NS	NS	NS							
Catechin	*	*	*	*	*	NS	NS	NS	NS	NS	NS						
Apigenin	*	*	*	*	*	NS	NS	NS	NS	NS	NS	NS					
Naringenin	*	*	*	*	*	*	*	NS	NS	NS	NS	NS	NS				
Kaempferol	*	*	*	*	*	*	*	*	*	NS	NS	NS	NS	NS			
Hesperidin	*	*	*	*	*	*	*	*	NS	NS	NS	NS	NS	NS	NS		
Vitamin C	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	
Naringin	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	NS

NS = $p>0.05$; * = $p<0.05$ (1) EPCG = Epicatechin gallate (2) EPGCG = Epigallocatechin gallate Q-3-G = Quercetin-3-Glucoside

Table 4.4 Total antioxidant capacities of flavonoids added to human plasma[1].

	n	TEAC (mmol/L) *(mean)*	SD	Difference from human plasma *P* (ANOVA)
Human plasma (control)	10	1.394	0.043	--
Plasma + 10 µmol/L Rutin[1]	3	1.397	0.032	NS
Plasma + 20 µmol/L Rutin	3	1.400	0.000	NS
Plasma + 50 µmol/L Rutin	3	1.443	0.041	NS
Plasma + 100 µmol/L Rutin	3	1.560	0.026	$p<0.001$
Plasma + 10 µmol/L Quercetin[2]	3	1.423	0.015	NS
Plasma + 20 µmol/L Quercetin	3	1.427	0.020	NS
Plasma + 50 µmol/L Quercetin	8	1.498	0.031	$p<0.001$
Plasma + 100 µmol/L Quercetin	10	1.637	0.041	$p<0.001$
Plasma + 100 µmol/L Kaempferol	3	1.597	0.020	$P<0.001$

[1] TEAC = 1.39 + 0.00241 x (quercetin concentration), $R^2 = 98.9\%$, $p<0.001$.

[2] TEAC = 1.38 + 0.00171 x (rutin concentration), $R^2 = 95.3\%$, $p = 0.004$.

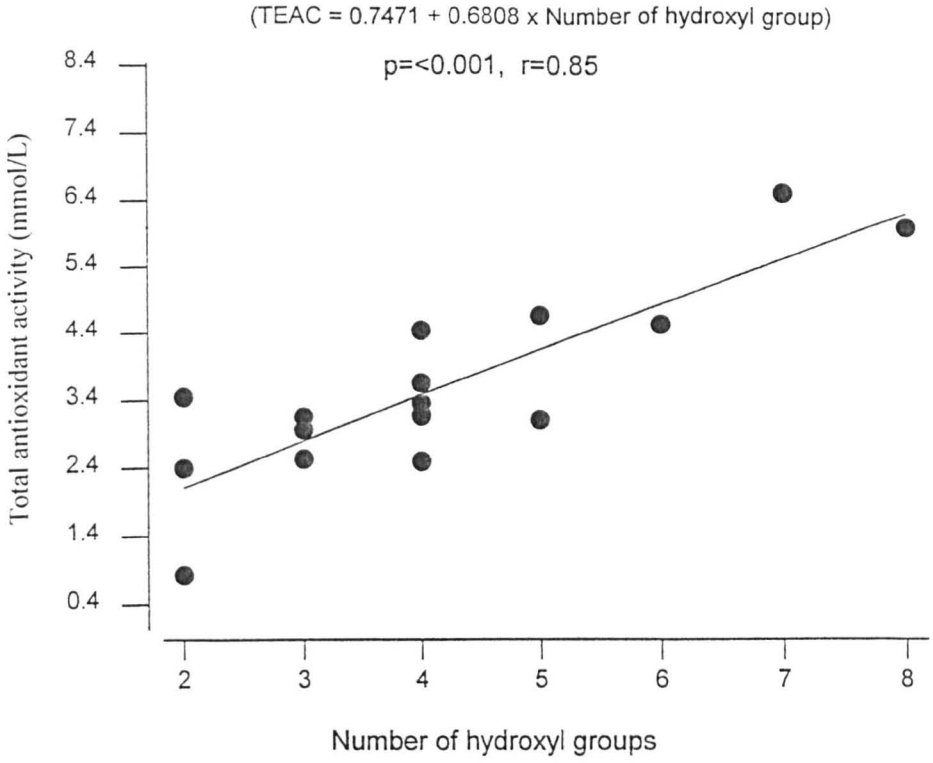


Figure 4.1 Effect of the number of hydroxyl groups in total antioxidant capacity (TEAC mmol/L) of flavonoids.

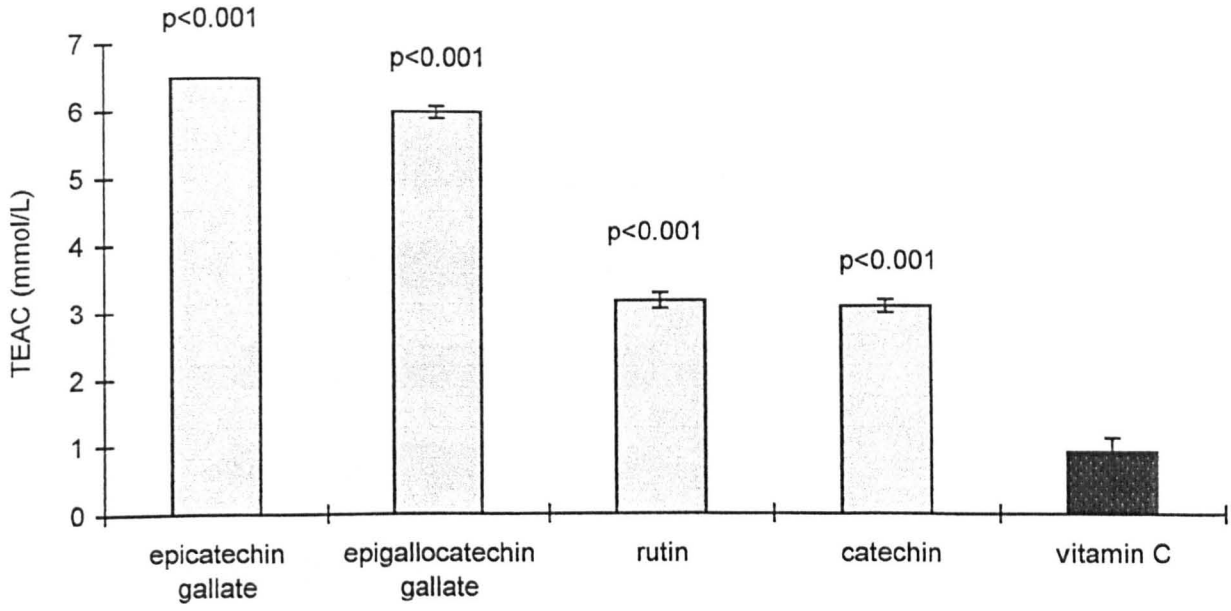


Figure 4.2 Comparison between the total antioxidant activities of vitamin C, catechins and rutin (tea, major sourse) (mean±SD)

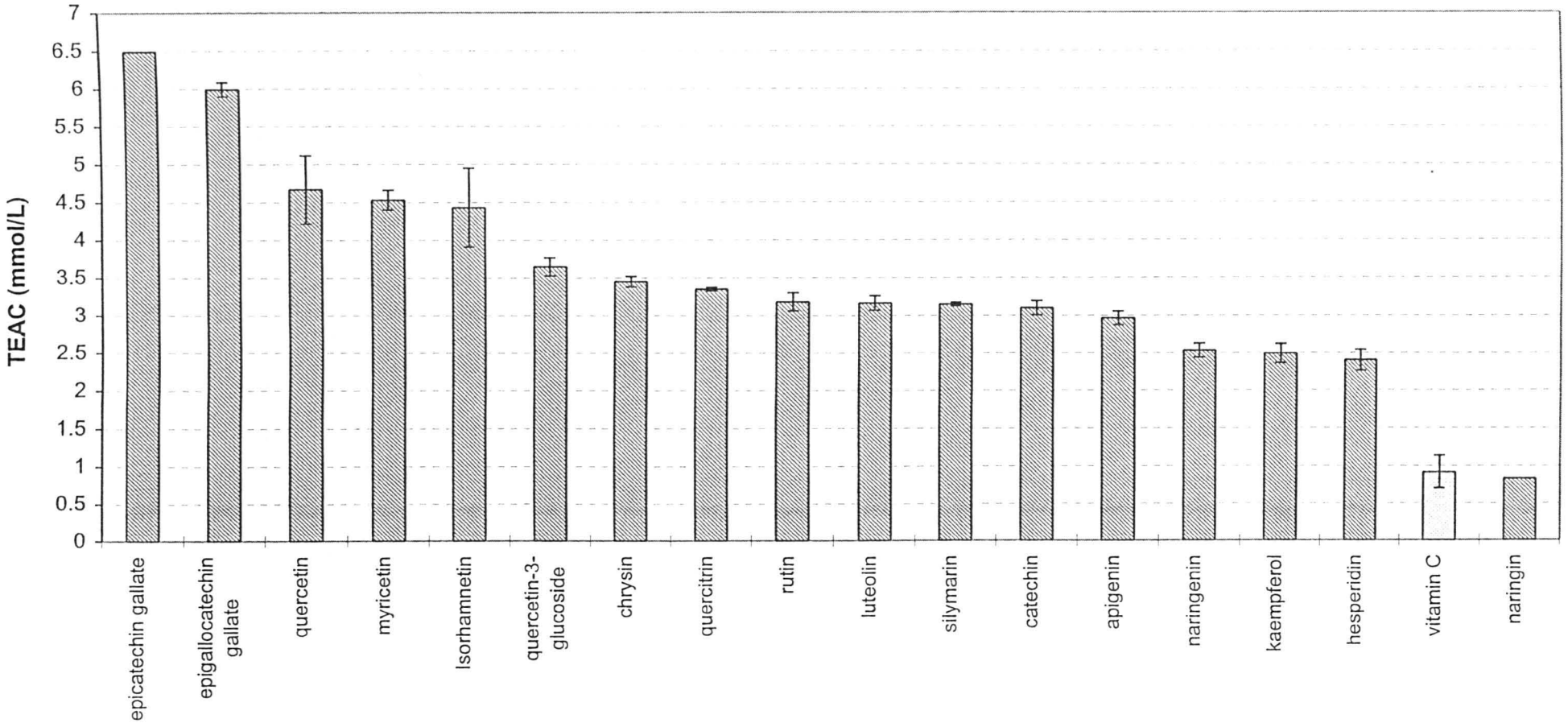


Figure 4.3 Total antioxidant capacity of flavonoids and vitamin C (mean±SD)

CHAPTER 5: Absorption of pure quercetin aglycone in humans

5.1 Introduction

The present study was designed to determine absorption of quercetin aglycone by human gut, with a view to undertaking a quercetin supplementation study.

Quercetin (3,5,7,3',4'pentahydroxyflavon) is one of the strongest antioxidant flavonoids, with a carbonyl group in C ring in position 3, two hydroxy groups in ring A and two hydroxyl group (3',4') in ring B (**Figure 2.4** and **3.2**).

Quercetin was selected for this study because it is one of the most widespread flavonoids in fruit and vegetables, and its consumption is also inversely associated with coronary heart disease mortality (Hollman et al 1995). However, the absorption of quercetin in humans is unclear.

5.2 Experimental design

Three single pilot experiments were designed and two volunteers (male) participated after giving informed consent. Subject A was 27 years, 80.2 kgs in weight, and subject B was 28 years, 64 kgs in weight. They were both considered healthy, non smokers, taking no medication or special

supplementation. They were both gastroenterologists in the Royal Infirmary, Glasgow.

5.2.1 Oral administration

Subject A: The experiment was repeated twice under same conditions and called A1 and A2.

A1 - After fasting overnight 500 mg quercetin dihydrate (aglycone) powder obtained (6.2 mg/kg body weight) from Gee Lawson Nutritional, London, UK, #3836, was mixed in 200 mls drinking water and swallowed within 20 seconds.

A2 - For the second experiment on subject A, 483 mg (6.0 mg/kg) of quercetin aglycone was used (Gee Lawson Nutritional, London, UK, #3836). The protocol was otherwise the same as for experiment A1.

Heparinised venous blood was taken at baseline and 0.5, 1,1.5, 2, 3, 5, 6.5 hours after administration. Plasma was separated immediately after the blood was taken and stored at –70°C until measurement. Duplicate samples were prepared for measurement of total antioxidant capacity by TEAC asay and HPLC analysis of quercetin. Urine was collected at baseline and 1, 2, 3, 5, 6.5 hours after administration of quercetin powder. Thimerosal (0.1g) (Sigma Chemicals Co Ltd, Irvine, Scotland, #T-5125) was added in urine bottle before freezing at –

70°C as an antibacterial to prevent bacterial degradation of quercetin before measurement. The quercetin aglycone powder was analysed for purity.

Subject B: Quercetin dihydrate aglycone (112 mgs) (1.8 mg/kg body weight) from Gee Lawson Nutritional, London, UK, #3836, was dissolved with 10 ml vodka (Smirnoff, 50% ethanol, produced from grain) by heating for 15 seconds in a microwave and swallowed within 20 seconds. Heparinised blood was collected in baseline and 0.5, 1, 1.5, 2, 3, 4.15 ,6.25 hours after administration. Urine was collected at baseline, and between baseline and 6.25 hours. The protocol was the same as for subject A.

5.2.2 Materials and methods

Explained in detail in Chapter 2, part 2.4 (total antioxidant activity of plasma) and part 2.5 (measurement of flavonoids in plasma and urine) of the thesis.

5.3 Results

Administration of pure quercetin aglycone in this study was well tolerated by both subjects. No effects were detected by the subjects. Total antioxidant capacity of plasma increased in one experiment (A2) from 0 (baseline) to 120 minutes after administration of quercetin aglycone **(Table 5.1, Figure 5.1)** but fell in two (A1, B), and there was no evident linear relation with time 0 (baseline) to 240 minutes.

After oral administration of 500 mg, 483 mg and 112 mg of pure querecetin in the three experiments no measurable plasma concentrations could be detected at any time point (limit of detection < 3 ng/ml), nor was any quercetin found in urine at any time (**figure 5.2**).

5.4 Discussion

This study was designed to establish the absorption of pure quercetin in humans but no detectable quercetin was found in urine and plasma in the first three experiments. This study was therefore abandoned.

Two previous published reports have used urine measurements to detect quercetin absorption. After oral administration of 4 g pure quercetin or 100 mg single intravenous quercetin (60 mg/kg body weight), Gugler and Co-workers (1975) found no measurable quercetin in plasma or urine. Their method of analysis in urine was not sensitive enough, (>200 μg/24 h urine sample). Hollman et al (1995) estimated 24% absorption of orally administrated quercetin aglycone (1.4 mg/kg body weight) while 52% for quercetin glucoside from onion (two fold more than aglycone). This assay was much more sensitive (5 ng/g urine). In the present study we used a highly sensitive flurometric HPLC analysis (< 3 ng/ml plasma, or urine) and still none could be detected.

One possible limiting factor for finding quercetin in human plasma is the conjugation form of quercetin. Kuhnau (1976) considered that aglycones are able to pass the gut wall, and that flavonoids present in foods cannot be absorbed from the small intestine because they are bound to sugars as glycosides. This view is clearly incorrect. Our results, and those of Nieder (1991); Hollman (1995); Aziz et al (1998) and McAnlis et al (1998) have all shown good absorption of quercetin glycoside from food sources (onions).

The measurements of TEAC in the present study cannot be considered statistically robust, but could suggest a rise in antioxidant capacity up to 120 minutes (**Figure 5.1**), which would coincide with peak quercetin absorption from food sources (McAnlis et al 1998). These results seem unlikely to be attributable to a rise in plasma quercetin of less than 1 ng/ml. It remains possible that the plasma quercetin results were artificially reduced, e.g. by deterioration of samples, although no obvious reason can be offered. The plasma quercetin measurements were made 8 weeks after the experiment but this should be within the safe period for storage at $-70^{\circ}C$, which has been established from work in Dr Crozier's laboratory (Crozier et al 1997a). It would be reasonable to undertake more detailed evaluation of the absorption of pure quercetin and its effect on antioxidant capacity. Because of lack of absorption of quercetin aglycone, it was decided to proceed to a diet study, based on onions and tea as rich sources of absorbable quercetin.

Table 5.1 Total antioxidant capacity of human plasma after administration of pure quercetin aglycone

	TEAC (mmol/l)		
	Subject A_1*	Subject A_2**	Subject B***
Supplement (quercetin aglycone) (mg/kg body weight)	**(6.2)**	**(6.0)**	**(1.8)**
Baseline	1.63	1.52	1.53
30 (min)*	-	1.53	1.56
60 (min)	1.53	1.55	1.55
90 (min)	1.59	1.56	1.56
120 (min)	1.59	1.67	1.55
180 (min)	-	1.57	1.53
240 (min)	1.55	-	-

* Administered 500 mg oral pure quercetin aglycone dissolved in 200 ml water

** Administered 483 mg oral pure quercetin aglycone dissolved in 200 ml water

*** Administrated 112 mg oral pure quercetin aglycone dissolved in 10 ml Smirnoff 50% ethanol vodka

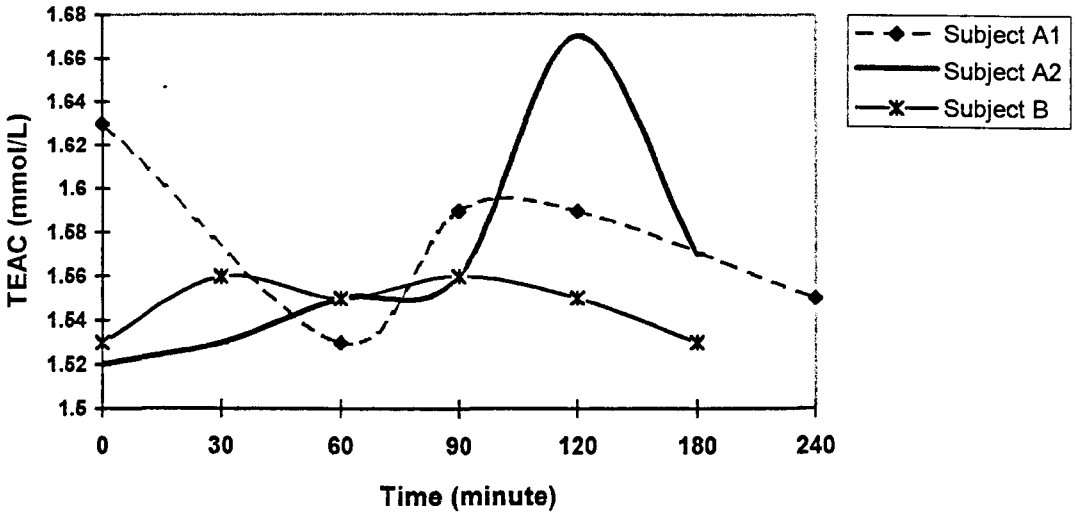


Figure 5.1 Total antioxidant capacity of human plasma after oral administration of quercetin aglycone in subject A1=6.2; A2=6.0 and B=1.8 mg/kg body weight

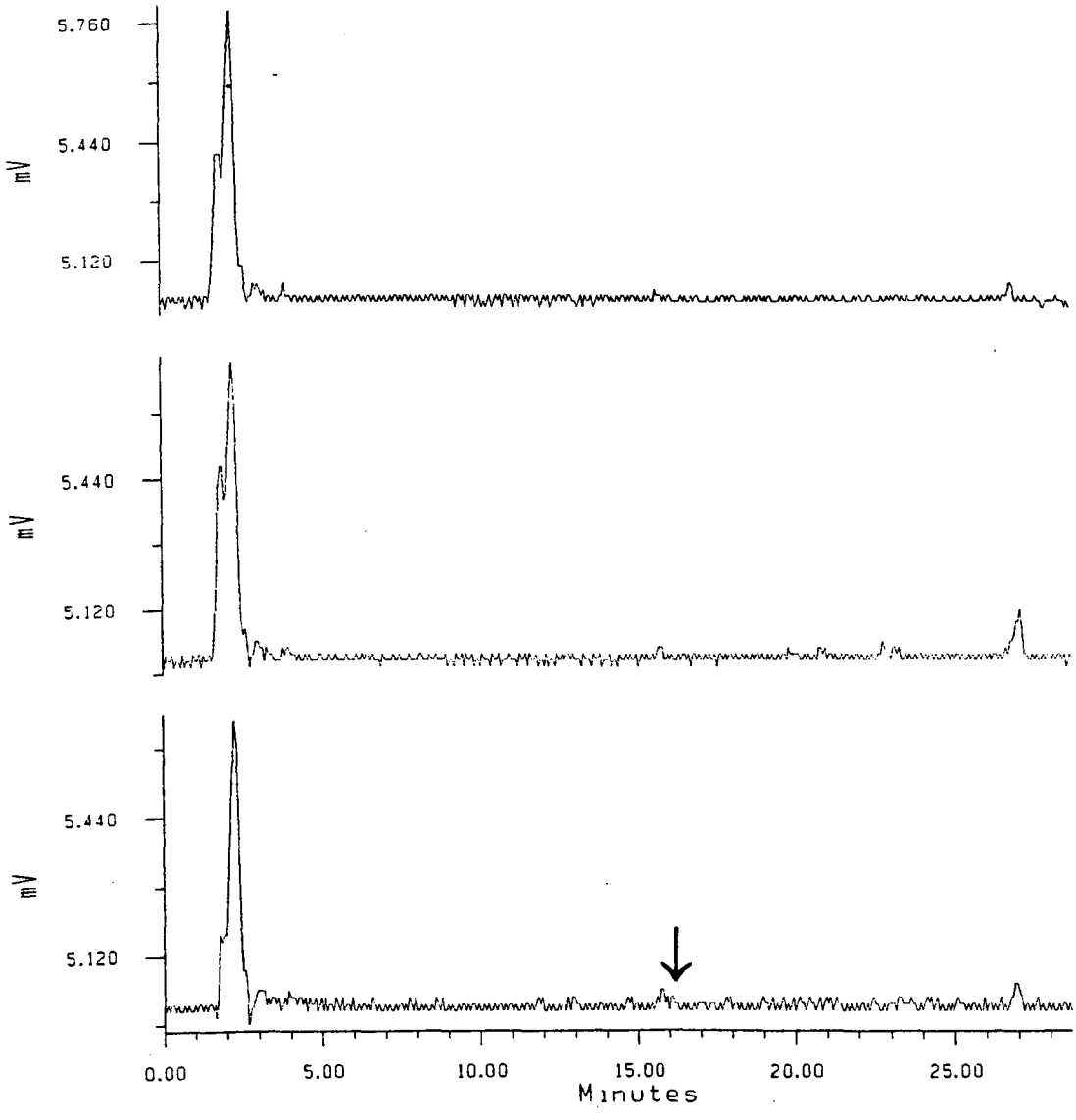


Figure 5.2 Quercetin plasma and urine analysis by HPLC, as shown in chromatogram an example in one of subjects, after oral administration of quercetin aglycone in subjects no detectable quercetin in plasma nor in urine were found. (A quercetin peak should be appear at 16 minute on chromatogram if absorbed).

Chapter 6: Dietary flavonols protect diabetic human lymphocytes against oxidative damage to DNA

(paper submitted to Diabetes, co-authors MEJ Lean, J Burns, D Talwar, N Sattar, A Crozier)

Abstract

Diabetic patients have reduced antioxidant defences and suffer from an increased risk of free-radical mediated diseases such as coronary heart disease. Epidemiological evidence has suggested that dietary flavonoids may protect against heart disease but a biological effect has yet to be demonstrated directly in humans. Ten stable NIDDM patients were treated for 2 weeks on a low flavonol diet and for 2 weeks on the same diet supplemented with 76-110 mg of flavonols (mostly quercetin) provided by 400 g onions (and tomato sauce) and 6 cups of tea daily. Freshly collected lymphocytes were subjected to standard oxidative challenge with hydrogen peroxide, and DNA damage was measured by single cell gel electrophoresis. Fasting plasma flavonol concentrations (measured by HPLC) were 5.6 ± 2.9 ng/ml on the low flavonol diet which increased twelve-fold to 72.1 ± 15.8 ng/ml on the the high flavonol diet ($p<0.001$). Oxidative damage to lymphocyte DNA was 220 ± 12 on an arbitrary scale 0 to 400 units on the low flavonol diet and 192 ± 14 on the high flavonol diet ($p=0.037$). This decrease was not accounted for by any change in the measurements of diabetic control (fasting plasma glucose or fructosamine), nor by any change in the plasma levels of known antioxidants including vitamin C, carotenoids, tocopherols, urate, albumin and

bilirubin. In conclusion we have shown, a biological effect of potential medical importance which appears to be associated with the absorption of dietary flavonols.

6.1 Introduction and Background

Diabetic patients, both IDDM and NIDDM, exhibit abnormal antioxidant status, auto-oxidation of glucose and excess glycosylated proteins (Young et al 1992; Davie et al 1992; Ceriello et al 1991; Jones et al 1985; Asayama et al 1993). Oxidative stress leads to tissue damage, increased reactive oxygen species, inactivation of proteins, fragmentation of DNA and tissue degeneration in diabetes mellitus (Wolffe et al 1991; MacRury et al 1993; Sinclair et al 1991; Dandona et al 1996). These factors are proposed to be important contributors to the development of the micro- and macro-vascular complications associated with diabetes. These complications include retinopathy, nephropathy and an increased risk of developing coronary heart disease (Sinclair et al 1991; Lyons et al 1991; Oberley et al 1988; Jennings et al 1991; Valezquez et al 1991). Dietary antioxidant compounds, including ascorbic acid and tocopherol, offer some protection against these complications through their roles as inhibitors of glycation and as free radical scavengers. In particular one study has reported that the flavonoid diosmin has the capacity to inhibit non-enzymatic protein glycation.

Flavonoids are a family of antioxidant polyphenolic compounds ubiquitously found in plants, typically as sugar conjugates. The family comprises of six sub-groups; flavonols, flavones, flavanones, isoflavones, anthocyanins and catechins (**Figure 1.1**). They are present in significant amounts in commonly consumed fruits and vegetables, particularly onions, apples and tomatoes, and beverages such as red wine and tea. Consumption of flavonoids, particularly the flavonol quercetin (3,5,7,3',4'-pentahydroxy-flavone) has been associated with a reduced incidence of heart disease and cancer (Hertog et al 1992; Hollman et al 1995; Knekt et al 1996). This protection is hypothesised to be due to the antioxidant properties of flavonoids. We have recently shown that flavonoids have very high antioxidant activities when compared to vitamin C, with quercetin and its conjugates consistently amongst the most potent (Noroozi et al 1998a). Although in vitro and epidemiological evidence indicate an important dietary role for flavonoids (Knekt et al 1996; Hertog et al 1993a; Keli et al 1996) debate has surrounded the issue of flavonol absorption. Current evidence suggests that while quercetin is poorly absorbed, its conjugates have been detected in significant quantities in plasma (Hollman et al 1996a & b).

The present study was designed to establish two factors, firstly whether dietary supplements of flavonol rich foods were absorbed consistently, and secondly whether they might have a biological effect in the protection against oxidative stress in NIDDM patients. The dietary supplement was of onions and tea on a setting of a low flavonol diet in a cross-over study. HPLC analysis was used to

determine the extent of flavonol absorption and a SCGE assay was used to determine the level of antioxidant defences. This was realised by measuring the oxidative damage incurred by fresh lymphocytes after both the low flavonol and supplemented flavonol diets. Possible confounding effects from other antioxidant systems were excluded by the measurement of known antioxidant vitamins, tocopherols, carotenoids and other compounds such as urate, albumin etc.

6.2 Subjects and study design

Patients with stable NIDDM, but healthy in other respects, were recruited from outpatient clinics. The inclusion criteria were NIDDM, no medication change during the study period, no vitamin supplements and not pregnant. Details of the patients and their pre-study diets (4 day weighed inventory) were analysed using COMPEAT **(Table 6.1)**. Of the ten subjects, 4 were treated with diet and oral hypoglycaemic agents (2 sulfonylureas, 2 biguanide) and 6 by diet alone. They were assigned, in random order, to follow either a high (supplemented) or low flavonol diet for 14 day periods in a crossover study. Two high flavonol diets were used, prepared as a palatable dish to be eaten in three equal portions with meals. Five subjects received a simple fried onion supplement (60.2 mg flavonols day^{-1}) and five subjects the same onion supplement with tomato ketchup and herbs (93.7 mg flavonols day^{-1}). Full details of the diet composition are given in Chapter 2 (part 2.7). All subjects also received a daily tea supplement containing

16.7 mg flavonols. Total flavonol supplements were thus 76.3 and 110.4 mg daily **(Table 6.2)**. Fasting blood samples and 24 hr urine collections were obtained at the baseline, low and high flavonol diet.

The protocol was approved by the Glasgow Royal Infirmary Medical Research Ethical Committee and all subjects signed a form of informed consent.

Dietary intervention (low and high flavonoid diet) are explained in detail in Chapter 2 (Part 2.7).

SCGE Assay, Endonuclease III assay, TEAC assay, HPLC analysis of flavonols, routine biochemistry methods are explained in detail in Chapter 2 (part 2.2 - 2.6).

Statistical methods are explained in Chapter 2 (part 2.8.3).

6.3 Results

Subjects reported high compliance with the low flavonol background diet throughout the study. The dietary supplements of onions and tea were well accepted and tolerated. Body weights did not change during the study (baseline 81.2 ± 3.5 kg, low flavonol diet 82.3 ± 3.8 kg, high flavonol diet 81.5 ± 4 kg).

Three subjects were smokers and did not change their habit during the study. The liver function tests judged by ALT, AST, bilirubin and ALP were essentially normal.

On the low flavonol diet, plasma flavonols were detectable in fasting plasma (above 1 ng/ml) in 3 subjects (mean 18.6 ng/ml) and undetectable in 7 subjects. The mean concentration for the whole group was 5.6 ± 2.9 ng/ml. On the high flavonol diets, fasting plasma flavonols were detectable in all subjects with a mean concentration of 72.1 ± 15.6 ng/ml for the whole group. The plasma concentration was numerically higher with the tomato ketchup and onion supplement than with onions alone, but the difference was not significant. Quercetin provided the greatest proportion of flavonols in the supplement **(Table 6.2)** and was also the major component of plasma flavonols **(Table 6.3)**. The supplements of 76.3 or 110.4 mg of flavonols (equivalent to 67-100.1 mg quercetin) on the background of a low flavonol diet therefore increased fasting flavonoid concentrations approximately twelve-fold.

Since the plasma and urine flavonols concentrations were not significantly different between the two high flavonol diets subjects were considered as a single high flavonol group. The scores from the SCGE give a measure of tissue protection against standard oxidative stress. The results showed a significant difference between the low and high flavonol diets supporting the hypothesis that a higher intake, and a greater absorption of flavonols are associated with a

significantly greater protection against oxidative stress at tissue level **(Figure 6.1, 6.2 & 6.3)**. Other measures used in this study to assess antioxidant effect were the endonuclease III assay, to detect endogenous oxidative damage to pyrimidine bases, and the TEAC assay to estimate the total antioxidant capacity of plasma. Neither of these tests gave significantly different results between the two diets and both showed relatively high variability **(Table 6.3 & 6.4)**.

Since many other factors may affect free radical antioxidant systems in the body, strenuous efforts were made to avoid any significant differences between the two diets in their content of other known antioxidant systems. The data in **Table 6.4** shows no change between high and low flavonol diets in any of the antioxidant vitamins or carotenoids, nor in selenium, superoxide dismutase, or glutathione peroxidase. There were no changes in plasma urate, albumin or bilirubin, all known to be powerful endogenous antioxidants. Plasma fructosamine was 320 μmol/l on the low flavonol diet, and 323 μmol/l with supplements, so the better antioxidant activity cannot be attributed to any improvement in diabetic control.

6.4 Discussion

Flavonols have been considered to be potentially beneficial components of fruits and vegetables for over sixty years. Their importance first came to light when a vitamin C sparing effect was observed, however although initially given the name

vitamin P they did not fulfil the criteria for essentiality (Rusznyak et al 1936). In vitro work has suggested a number of potentially important functions for flavonols. Their antioxidant activity is of particular importance, notably in the protection against LDL oxidation, an key process in the pathogenesis of artherosclerosis (De Whalley et al 1990). Recent work using HPLC has provided improved information about the flavonol content of foods. Available data from the Netherlands suggests that flavonols are present in the diet at levels in the order of 23 mg per day, mostly in the form of quercetin and largely obtained from tea (61%), onions (13%) and apples (10%) (Hertog et al 1992). Much larger daily intakes might be expected in high consumers of these food, and of specific varieties in particular as it appears that there are clear and consistent differences between the flavonol contents of distinct varieties of fruits and vegetables (Crozier et al 1997a).

Until recently there was very little information available on whether dietary flavonoids, particularly flavonols are absorbable. Early data suggested that the conjugated flavonols, in contrast to the aglycone, were precluded from intestinal absorption (Kuhnau et al 1976). However, acute dosing experiments have recently indicated the opposite ie greater absorption of conjugated flavonols and minimal absorption of the aglycone (Hollman et al 1996a; Aziz et al 1998; McAnlis et al, 1998). These acute experiments have shown an elevation of plasma flavonols for 1-5 hours after dosing. The present study is the first to examine the extended treatment of high flavonol supplements, and relate this to measurements of

protection against oxidative stress at a tissue level. Very clear evidence has emerged showing significant absorption of dietary flavonols, specifically quercetin and its conjugates. There is evidence of flavonol absorption in all 10 subjects with a mean increase in fasting plasma flavonol concentrations of approximately twelve fold from a relatively small supplement.

Several studies have demonstrated improved antioxidant defences in subjects given foods or diets which might contain increased flavonoids. However these studies are largely without evidence that flavonoids are absorbed and thus responsible for the improvement observed. In addition the potential confounding effects from other antioxidant factors may not have been rigorously excluded in all instances. Direct evidence on the biological effects of flavonoids is very limited. Maxwell et al (1994) demonstrated improved antioxidant status from the consumption of red wine, known to be a rich source of flavonoids. Red wines vary in their flavonol content from 4.6 to 41.6 mg/l, so may certainly contribute some antioxidant flavonols to the diet (McDonald et al 1998) but other phenolic in wines may be more quantitatively important than the flavonols. Ishikawa et al (1997) have recently found a reduction in LDL oxidation in subjects fed 750 ml black tea daily for 4 weeks. They showed absorption of catechins, a sub-group of the flavonoid family, and suggested that this may have accounted for the reduced LDL oxidation, although tea does also contains conjugated quercetin, myricetin and kaempferol.

The acute consumption study of McAnlis (McAnlis et al 1994) showed no effect from 225 g of onions on the resistance of plasma to copper induced oxidation. This test is similar to the TEAC assay used in the present study, which also showed no significant difference on the fasting antioxidant capacity of plasma between high and low flavonol diets over 28 days. These tests are relatively crude, and may not relate directly to free-radical mediated damage within cells. Our use of SCGE on fresh lymphocytes to assess the result of a dietary intervention was a novel approach and showed, at a tissue level, a significant increase in the protection against DNA damage from H_2O_2. We have previously employed the SCGE assay to study the antioxidant effect of pre-incubation with flavonoids including flavonols, and have found dose-dependent effects with all common flavonoids most being significantly more potent that vitamin C (Noroozi et al 1998 a & b)

There is growing awareness that free-radical processes may be of particular importance in the microvascular and macrovascular complications of diabetes (Gazis et al 1997). There is already abnormal antioxidant status in the pre-diabetic state of impaired glucose tolerance (IGT) and this may contribute to the high coronary heart disease risk in IGT (Vijayalingam et al 1996). Decreased lipid peroxidation and improved antioxidant status may be one mechanism by which dietary treatment contributes to the prevention of diabetic complications (Armstrong et al 1996). Sinclair et al (1992a) have reported that there is a negative

correlation between serum ascorbic acid and fructosamine concentration in diabetic patients with complications. This group has also reported a low concentration of plasma ascorbate in patients with type 2 diabetes mellitus consuming adequate dietary vitamin C, and suggested that this implies increased utilisation of vitamin C to inactivate free radicals. Dietary recommendations for diabetes encourage high fruit and vegetable intakes (Diabetes and Nutrition Study Group (DNSG), 1995). The present study provides further evidence to justify this recommendation, primarily aimed at reducing cardiovascular disease.

Evidence linking flavonoids with protection against cancers is weaker than that for cardiovascular disease in the general population (Hertog et al 1993a) although Dorant et al (1994a) found an inverse relationship between onion consumption and cancer risk - particularly stomach, colon and rectum. There is probably no major increase in cancer risk amongst diabetic patients, but these findings are consistent with previous studies which have shown diabetes to be a risk factor for cancer of the uterine corpus, similarly a positive association between prior diagnosis of diabetes was noted for kidney cancer and non-melanoma skin cancer in females (ÓMara et al 1985).

Very few studies in diabetic subjects have sought improvements from administration of known antioxidants although claims have been made for high dose vitamin C and E (Paolisso et al 1993). In SCGE studies we have shown that the effect of flavonols is additive to that of vitamin C and on this basis it would

seem appropriate to suggest that diets relatively high in flavonols as well as conventional antioxidant vitamins should be recommended for patients with diabetes. It might be hypothesised that diabetic patients with high intake of flavonol rich foods, and specifically onions, might be relatively protected against long term complications. Evidence for such an effect does not exist at present but appropriate analyses of large data bases might be encouraged to seek supporting evidence of this kind.

Table 6.1 Characteristics of 10 NIDDM patients (5 male, 5 female) and background daily nutrient intake assessed by four days weighed diet diary (2 weekdays, 2 weekend days)

Nutrient Intake	Group mean ±SD	Range
Age(years)	60.1±6.95	50 - 74
Height(m)	1.64±0.1	1.49 - 1.83
Weight(kg)	81.24±11.06	69.4 – 107.2
BMI(kg/m^2)	30.15±3.53	24.9 - 38.31
Duration of diabetes (years)	6±4	2-11
Energy (kcal)	1989.7±703.4	805 - 2897
Fat (%E)	38.4±6.18	32.30 – 53.30
Protein (%E)†	19.9±3.3	16.80 - 27.70
Carbohydrate (%E)	39.0±7.33	28.5 - 49.50
Ethanol (%E)	2.52±4.02	0.00 - 10.5
NSP‡ (g/day)	14.6±8.41	3.00 - 32.04
Iron (mg/day)	15.97±7.24	4.75 - 31.27
Copper (mg/day)	1.26±0.44	0.52 - 1.99
Selenium (µg/day)	40.9±27.21	14.15 - 96.72
Vitamin C (mg/day)	56.7±33.37	22.00 – 123.00
Vitamin E (mg/day)	4.85±2.98	1.21 – 9.80
Vitamin A (µg/day)	559.2±275.31	76.0 - 928.00
Tea (ml/day)	717.2±498.05	0 – 1425
Onions (g/day)	4.15±4.5	0 – 12.5

† %E = percent of total daily energy intake ‡ NSP = non starch polysaccharides

"None of the patients had clinically detectable micro or macro vascular complications of diabetes."

Table 6.2 Flavonol and vitamins content of food supplements (tea and onion dish) used for the high flavonol diet

	Tea 6 mug (1500 mls)		Plain Onion (400 g)		Onions and tomato ketchup and herb (400 g)	
Flavonols and vitamins	µg/ml	mg/day	µg/g	mg/day	µg/g	mg/day
Vitamin A (retinol)	0	0	<0.02	<8g/day	<0.02	<8g/day
Vitamin E (α-tocopherol)	0	0	n.d.	n.d.	n.d.	n.d.
Vitamin C	0	0	3.6	14.4	1.1	4.4
Free quercetin	0.41		5.4		4.20	
Conjugate quercetin	7.04		136.9		221.07	
Total quercetin	7.08	10.0	142.0	57.0	225.37	90.15
Free kaempferol	0.18		0.03		0.03	
Conjugate kaempferol	3.24		0.66		0.98	
Total kaempferol	3.26	4.89	0.72	0.7	1.01	0.41
Free myricetin	n.d.		n.d.		n.d.	
Conjugate myricetin	0.79		n.d.		n.d.	
Total myricetin	0.79	0.78	n.d.	0	n.d.	0
Free isorhamnetin	n.d.		0.19		0.17	
Conjugate isorhamnetin	n.d.		6.03		7.59	
Total isorhamnetin	n.d.	n.d.	6.21	2.5	7.78	3.11
Total flavonols	11.14	16.7	148.94	60.2	234.2	93.67

Total daily intake of flavonols provided by test diet with onion, tomato ketchup, 110.37 mg/day and with plain onion, 76.3 mg/day.

Table 6.3 Plasma and urine flavonol responses of diabetic patients (NIDDM) to high flavonol diet, SCGE assay to measure protection of fresh lymphocytes against H_2O_2 damage to DNA and endonuclease III assay for endogenous DNA damage analysis

	Low flavonol diet	**High flavonol diet**	***P*-value**
Fasting plasma Quercetin (ng/ml)	5.6±2.9	67.8±15.2	<0.005
Fasting plasma total flavonols (ng/ml)	5.6±2. 9	72.1±15.7	<0.005
24 h urine quercetin concentration (ng/ml)	12.5±5.2	112.4±17.7	<0.0005
24 h urine total flavonols concentration (ng/ml)	15.2±6.2	148.9±20.7	0.0001
24 h urine quercetin excretions (μg/day)	17.3±7.5	218.0±52.5	<0.005
24 h urine total flavonol excretion (μg/day)	21.2±9.0	281.8±59.1	<0.005
SCGE (out of 400) †	220.0±12.0	191.5±13.5	0.037
Endonuclease III (out of 400)	82.4±4.5	91.4±10.9	0.42

Data are means ± SE (n = 10) † Single Cell Gel Electrophoresis (SCGE)

Table 6.4 Plasma and urine measurements of antioxidant factors on high and low flavonol diets

	Low flavonol diet	High flavonol diet	*P*-value
Superoxide dismutase activity (units/ml)	0.027±0.01	0.051±0.021	0.30
Gluthathione peroxidase (units/L)	210.30±12.78	213.0±8.83	0.86
Selenium (μmol/l)	1.278±0.095	1.238±0.084	0.36
$TEAC^{+}$ (mmol/l)	1.40±0.03	1.44±0.03	0.22
Plasma Albumin (g/l)	45.10±0.82	45.86±0.64	0.69
Urine Albumin (mg/l)	13.38±4.60	16.40±6.40	0.12
Plasma globulin (g/l)	30.78±1.21	30.0±1.03	0.74
Total plasma protein (g/l)	72.80±3.67	76.0±1.65	0.86
Plasma bilirubin (unit/l)	13.78±2.76	15.57±3.32	0.83
Fructosamine (μmol/l)	320.4±22.4	323±20.1	0.72

Data are means ± SE (n = 10) $^{+}$=Trolox equivalent antioxidant capacity of plasma

Tables 6.4 (continued) Plasma antioxidant vitamins and carotenoids on low and high flavonoid diets

	Low flavonoid diet	High flavonoid diet	*P*-value
Vitamin A (μmol/l)	2.62±0.48	2.74±0.43	0.28
Vitamin C (mmol/l)	35.7±3.43	30.10±3.2	0.13
Vitamin E (μmol/l)	42.0±5.52	45.70±6.11	0.11
Vitamin E/chol †	6.69±0.67	7.24±0.57	0.15
β-carotene (μmol/l)	0.16±0.03	0.16±0.04	0.95
β-carotene/chol †	0.028±0.007	0.027±0.008	0.47
β-cryptoxanthine (μmol/l)	0.04±0.01	0.04±0.01	0.39
β-cryptoxanthine/chol†	0.006±0.001	0.006±0.001	1.00
Lycopene (μmol/l)	0.197±0.039	0.244±0.059	0.22
Lycopene/chol †	0.034±0.007	0.042±0.011	0.22
Total carotenoids(μmol/l)	0.605±0.067	0.652±0.103	0.44
Total carotenoids/chol†	0.104±0.016	0.112±0.020	0.38
Lutein (μmol/l)	0.16±0.02	0.15±0.02	0.57
Lutein/chol †	0.03±0.004	0.03±0.005	1.00
α-carotene (μmol/l)	0.05±0.008	0.05±0.01	0.62
α-carotene/chol †	0.009±0.002	0.009±0.002	0.17
cholesterol (mmol/l)	6.32±0.55	6.32±0.63	1.00

Data are means ± SE (n = 10) †Ratio to plasma cholesterol ($x10^{3}$ = mmol/l)

Laboratory Reference ranges: Vitamin A (1.4 - 2.6 μmol/l), Vitamin E (2 2-37.2μmol/l),Vitamin C (11-114 μmol/l), Lutein (0.15-0.37 μmol/l), Lycopene (0.19-0.55 μmol/l),α-carotene (0.03-0.11 μmol/l), β-carotene (0.18-0.58 μmol/l), β-cryptoxanthine (0.14-0.36 μmol/l).

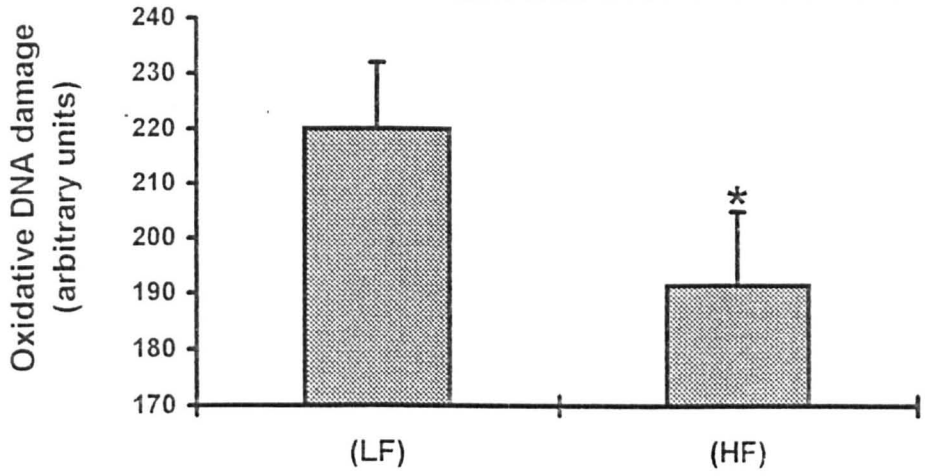


Figure 6.1 The effect of high flavonoid diet (HF) and low flavonoid diet on protection against oxidative DNA damage in diabetic patients (NIDDM) in the SCGE assay analysis by paired t-test, mean± SE for n=**10.** *, $P < 0.05$.

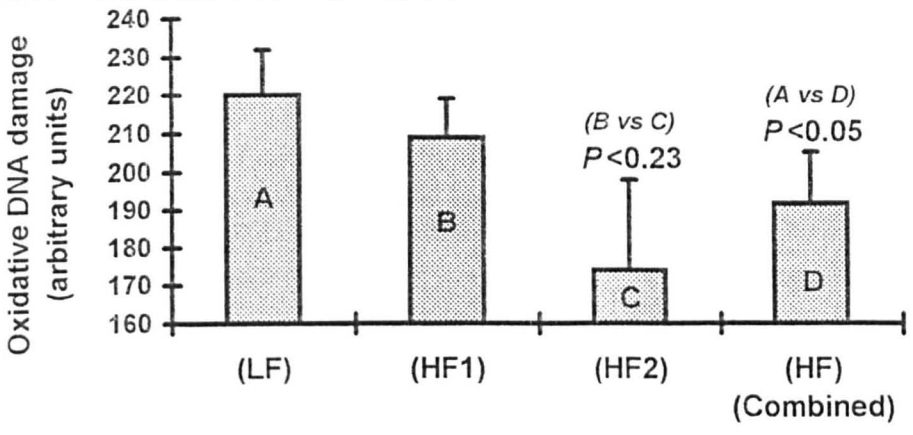


Figure 6.2 The effect of low (LF) and high flavonoid diet (HF1)= (onion+tomato ketchup + herb), (HF2)= (plain onion) on protection against oxidative DNA damage in lymphocytes of diabetic patients (NIDDM) in the SCGE assay analysis. n=5 (B&C); n=10 (A&D).

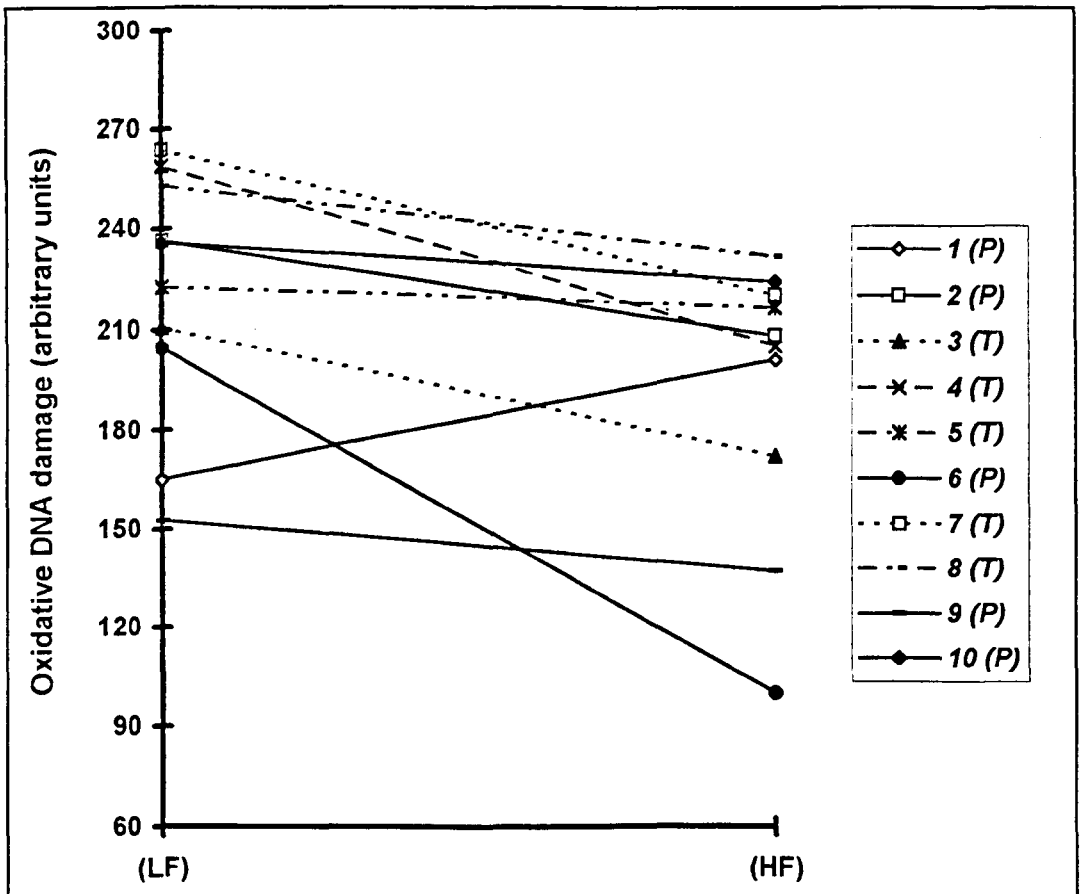


Figure 6.3 Endogenous protection against oxidative DNA damage in lymphocytes, on low (LF) and high flavonols diets (HF) in random order. (P)=plain onion, (T)=onion and tomato ketchup and herb, mean ±SE for n=10, $P<0.05$.

Chapter 7: Prediction of dietary flavonol consumption from fasting plasma concentration or urinary excretion

(paper submitted to American Journal of Clinical Nutriton, co-authors: Lean MEJ, Burns J, Crozier A, Kelly I

7.1 Introduction

The flavonols belong to the large group of flavonoids, and quercetin is the major representative of the flavonols subclass, whose chemical structures depend on the differences in the 3', 5' and 3 positions of the band C rings (**Figure 1.1** and **2.4**). Major dietary sources of flavonols are onions, kale, brocolli, apples, cherries, berries, tea (Hollman 1997; Hertog 1993b;Hertog et al 1992).

Little information is available on the absorption, metabolism and excretion of flavonols in humans, although there is information about the flavonoid contents of certain foods, for example those analysed by Hertog et al (1992), Crozier et al (1997a). Estimates of total flavonoid consumption vary from 29 mg/day (Hertog et al 1993a) to 1 g/day, (Kühnau 1976). These estimates are based on incomplete analysis of a relatively small number of foods applied to dietary records. Based on the work of Gugler et al (1975), who observed no absorption of the quercetin aglycone by human gut, Kühnau (1976) concluded that conjugate flavonoids in foods are not absorbed and only aglycones are absorbed

in the human gut. However, this conclusion was clearly incorrect: the bioavailability of flavonoids has more recently been assessed using modern HPLC methods (Hollman 1997 and 1995), (Paganga et al 1997), (Hertog and Hollman 1996), (McAnlis et al 1998), (Aziz et al 1998) and have all shown definite but variable absorptions from foods. Hollman et al (1995) found much lower absorption of aglycone quercetin than glycosides from onions (24% versus 52%), but most flavonoids in foods are in conjugated form.

This study focusses on the relationship between flavonols intake (on test diets designed to have either low or high contents), urine excretion and plasma concentration of flavonols, with the aim of establishing a biomarker for the flavonol content of the habitual diets of free-living subjects.

7.2 Subjects and study design

Three test diets were designed. The basis was dietary advice to follow a low flavonoid diet, assumed to contain no flavonols. Two high flavonol diets both contained tea (6 cups daily), and an onion dish or an onion/tomato ketchup/herb dish, taken in 3 equal portions with meals. (Full details in **table 6.2** and part 6.2 (Chapter 6).

Ten NIDDM subjects were studied, allocated in 14-day periods to consume the low flavonol diet, or to one of the two high flavonol diets, in randomised crossover design. The characteristics of the subjects are shown in **Table 6.1**. Seven day weighed diet records at baseline (i.e. on each subject's habitual diet) were analysed by a research dietitian using the COMPEAT nutrient database (McCance and Widdowsons). A 24 h urine collection and fasting venous blood sample were taken at baseline (on their usual diet) and at the end of each 14 day test period.

Test diet, flavonols analysis and statistical analysis methods are explained in detail in Chapter 2 (part 2.7, 2.5 and 2.8.4).

7.3 Results

High flavonol supplements

Total flavonols in the high flavonol supplements showed the highest concentration of flavonoids in onion combined with tomato ketchup (234.2±5 μg/g) followed by plain onion (148.9±8.5 μg/g). The tea contained 11.1±0.4 μg/ml. Most of the flavonols were quercetin and most of flavonoids in the supplements were quercetin conjugates (96-98%) **(Table 7.1)**.

Daily flavonol consumption

Total daily intake of flavonol on the test diets (from supplements plus tea) was calculated at 110.4 mg with tea, onion and tomato ketchup and herb supplement, while from plain onion test diet consumption was 76.3 mg. The major flavonol in the two high flavonoid test diets was quercetin, (90.1 and 57.0 mg respectively). Tea in the high flavonols test diets provided 16.7 mg total flavonols, 10.8 mg quercetin.

Total flavonols

Fasting plasma and urinary flavonols concentration of individual or all subjects are shown in (**Table 7.2 and 7.3**) on low and high flavonol diets. In urine the percent of flavonols present as conjugates on baseline, low and high flavonoids diet were 82.3%, 67% and 87.4%. In plasma 100% of flavonols were in conjugated forms. Fasting plasma and urinary flavonols concentrations were highly correlated.

Both fasting plasma and urinary (**Figure 7.2**) flavonol concentrations were highly significantly related to dietary intake. For the purposes of determining dietary intake, however, urinary values appear marginally better (r^2= 71.8% *vs* 55.6%, both $p < 0.001$). There was little additional benefit from using both parameters in an equation to predict dietary intake derived from multiple regression analysis (r=72.4%, $p < 0.001$).

Quercetin

Fasting plasma and urinary concentrations are shown in **Table 7.4**. These values were highly correlated (**Figure 7.3**). Both plasma and urinary flavonol (**Figure 7.5**) values were highly significantly related to dietary intake. For the purposes of determining dietary intake, as with total flavonols, urinary values appear marginally better (r^2= 66.3% *vs* 55.3%, both $p < 0.001$). There was little additional benefit from employing both parameters in a multiple regression equation (r=69.6%, $p < 0.001$).

Total 24 hour urine excretion of flavonols

It might be expected that total urinary excretion should give a better prediction of intake than urine concentration. However, in the present study on free living diabetic subjects this did not prove to be the case. Thus regression coefficients for 24 h urinary excretion of total flavonols (r=0.728, p<0.001) (**Figure 7.2**) and for 24 h urinary quercetin excretion (r=0.681, p=0.001) (**Figure 7.4**) were weaker than those for urinary concentrations. Tests for completeness of urine collection were not employed. Urine volume ranged from 0.81-2.64, at baseline, 0.86-2.48 on low flavonol diet and 0.87 - 4.45 on high flavonol diet.

Flavonol intake on habitual diets

The measurements of fasting plasma flavonols (and quercetin), and of urinary flavonols, made at baseline on each subject's habitual diet, were applied to the

regression equations to estimate the flavonol consumption of each subject on their habitual diet, shown in **Tables 7.3 and 7.4.**

The average flavonol intake on the baseline diet, estimated from a regression equation based on fasting plasma flavonols, was 35.2±3.5 SD mg/day. Estimated from urine concentration, the flavonol concentration was 33.2±7.2 SD mg/day. There was a wide range of values from 17-50 mg/day based on plasma concentrations, 18-82 mg/day from urine measurements.

Estimates of quercetin intake from fasting plasma quercetin (31.9±SD mg/day) and from urine concentration (41.2±SD mg/day) were closely related to flavonols intakes (**Table 7.4**).

Dietary analyses

Table 6.1 shows the nutrient analyses of subjects at baseline, during the 4 days immediately before the measurements were made. The mean and range for key nutrients in these diabetic patients are very similar to those of the general population. In particular, the figures for dietary fibre, vitamin C and vitamin E do not point to an unusual consumption of fruit or vegetables in these diabetic subjects. There were no statistically significant relationships between plasma flavonols and quercetin and dietary intakes of total fruit and vegetable intake, total vegetable intake, total potato intake, total fruit intake or total tea intake at

baseline. The highest correlations observed were with total vegetable intake and plasma flavonols (r=0.38, p=0.40) or plasma quercetin (r=0.36, p=0.43).

7.4 Discussion

The baseline measurements, reflecting the habitual diets of free-living adults revealed detectable flavonols in both fasting plasma and in urine of 8/10 subjects. The main contributor to dietary flavonols was quercetin at 92.4% of total flavonols in diet and both urine and plasma measurements. For both total flavonols and quercetin there was a close correlation between the fasting plasma and 24 h urinary concentration so regression equations employing plasma or urine gave very similar results, and either could be employed to estimate dietary exposure.

The average flavonol intake estimated in the present study (in Glasgow) is higher at 35 mg/day than in the countries, except Japan and Croatia, estimated from food records by Hertog et al (1995) and quercetin intake in the present study (32 mg/day) is more than any of the 8 countries presented in **Table 7.5**. The subjects in the present study were not necessarily representative of the adult Scottish population and the influence of their NIDDM cannot be assessed, but they were free living adults, with diet compositions very similar to those of the general population. There is no *priori* reason to expect NIDDM to affect the

results, although vitamin C levels may be low in diabetic subjects (Sinclair et al 1994). The consumption of fruits and vegetables were low by international standards, so the flavonoid intakes in other countries are likely to be considerably higher. Given their powerful antioxidant actions, the large range in flavonol consumptions found in the present study (18-82 mg/day), and the wide range in plasma concentrations (0-44 ng/mL), point to the possibility of a wide range in dietary antioxidant protection between individuals with different diet compositions.

The method we have developed offers the potential to make simple estimates of flavonols consumption of free-living individuals on the basis of a urine collection, or from a fasting blood sample. The failure of a figure of total 24 h urine excretion to give a better prediction of intake probably illustrates the difficulty in obtaining complete urine collections, and it is interesting that the urine flavonol concentration gave such good results. In routine research, a fasting plasma sample is likely to be a more widely applicable and reliable test. For application in the general population the results of the present study should ideally be supported by similar data in non-diabetic individuals. The data in this small study of 10 subjects offers some hope that plasma or urinary flavonol measurement might prove a useful biomarker for vegetable, or fruit and vegetable intake. The present study suggests that this approach is feasible, but relies on the "low flavonoid" diet having an assumed zero content of the flavonols. A larger range of intakes will need to be studied to establish a true

dose response, and to investigate if this saturation kinetics, develop at high intakes.

Table 7.1 Daily flavonol content of test diets containing 400 g onion or 400 g onion plus tomato ketchup and herb plus 1500 ml/L of tea/day. Data are presented as means of 3 triplicate analyses

High Flavonoid diet	Quercetin (mg)		Myricetin (mg)		Kaempferol (mg)		Isorhamnetin (mg)		Total flavonol content (mg/d)	Total quercetin content (mg/d)	Conjugated flavonoids	
											(mg/d)	% of total flavonoids
	Free	Conj	Free	Conj	Free	Conj	Free	Conj				
Onion + tomato Ketchup + tea*	1.7	88.4	nd[+]	nd	nd	0.4	0.1	3.0	93.6	90.1	91.8	98%
Plain onion**	2.2	54.8	nd	nd	nd	0.7	0.1	2.4	60.2	57.0	57.9	96%
Tea	0.1	10.7	nd	1.2	0.3	4.8	nd	nd	17.1	10.8	16.7	97.7%

*(Total daily intake plus tea = 110.4 mg/d)
**(Total daily intake plus tea = 76.3 mg/d)
[+] = none detected

Table 7.2 Plasma and urine flavonol concentrations of diabetic patients (NIDDM) on low and high flavonol diets

	Low (0) flavonol diet n = 10	**76 mg/day flavonol diet** n = 5	**110 mg/day flavonol diet** n = 5
Fasting plasma Total flavonols (ng/ml)	5.6±2. 9	52.2±12.4	91.9±27.6
Fasting plasma Quercetin (ng/ml)	5.6±2.9	48.3±11.9	87.3±26.7
24 h urine quercetin concentration (ng/ml)	12.5±5.2	93.7±15.0	131.2±31.8
24 h urine flavonols concentration (ng/ml)	15.2±6.2	126.5±15.5	171.2±37.9
24 h urine quercetin excretions (μg/day)	17.3±7.5	186.8±50.5	262.0±80.1
24 h urine total flavonol excretion (μg/day)	21.2±9.0	246.9±57.1	275.6±82.7

Data are means ± SEM

Table 7.3 Prediction of dietary flavonols consumption from fasting plasma or urine concentration on baseline diets

Subjects	fasting plasma flavonols (ng/mL)	estimated flavonols intake (mg)*	24 h urine flavonols excretion (μg)	urine flavonols concentration (ng/mL)	estimated flavonols intake (mg)**
1	43.7	50.4	300.7	151.9	81.9
2	22.1	33.9	46.1	37.5	24.2
3	0	17	16.8	12.1	11.4
4	40.2	47.7	36.4	25.1	18
5	0	17	50.7	31.7	21.3
6	30.5	40.3	260.1	122.7	67.2
7	23.3	34.8	122.4	46.4	28.7
8	31.1	40.8	69.4	39.4	25.2
9	26.8	37.5	39.1	48.9	30
10	20.5	32.7	31.3	37.3	24.1
Mean±SEM	23.8±4.6	35.2±3.5	97.3±32	55.3±14.2	33.2±7.2

*(flavonols intake (mg) = 17.0+0.764 x fasting plasma flavonols concentration) r=0.74, p<0.001
**(flavonols intake (mg) = 5.34+0.504 x urine flavonols concentration) r=0.847, p<0.001

Table 7.4 Prediction of dietary quercetin consumption from fasting plasma or urine concentrations on baseline diets

Subjects	fasting plasma quercetin (ng/mL)	estimated quercetin intake (mg)*	24 h urine quercetin excretion (μg)	urine quercetin concentration (ng/mL)	estimated quercetin intake (mg)**
1	41.7	45.8	250.1	126.3	78
2	22.1	31.7	36.8	29.9	23.9
3	0	15.8	16.8	12.1	13.9
4	40.2	44.7	25.8	17.8	17.1
5	0	15.8	28.6	17.9	17.1
6	21.8	31.5	203.3	95.9	60.9
7	23.3	32.6	72.0	27.3	22.4
8	28.2	36.1	39.0	22.2	19.6
9	26.8	35.1	28.8	35.9	27.2
10	20.5	30.5	22.6	26.9	22.2
Mean±SEM	22.5±4.4	31.9±3.2	72.4±26.4	41.2±12.1	30.2±6.8

*(quercetin intake = 15.8+0.719 x fasting plasma quercetin) r=0.744, p<0.001
**(quercetin intake = 7.10+0.561 x urine quercetin concentration) r=0.814, p<0.001

Table 7.5 Comparison between estimation of flavonol and quercetin intake estimated from diet records in middle aged men in the Seven Countries Study (Hertog et al 1995) and in the present study (Glasgow)

	Quercetin intake (mg/d)	**Flavonol and flavonone intake (mg/d)**
Finland	6	6
USA	11	13
Serbia	10	12
Greece	15	16
Italy	21	27
The Netherlands	13	33
Croatia	30	49
Japan	31	64
Glasgow (present study)	**31.9**	**35.2**

Predicted from flasting plasma flavonols, $n = 10$, age 60±69.5 *y*, 5 male and 5 female

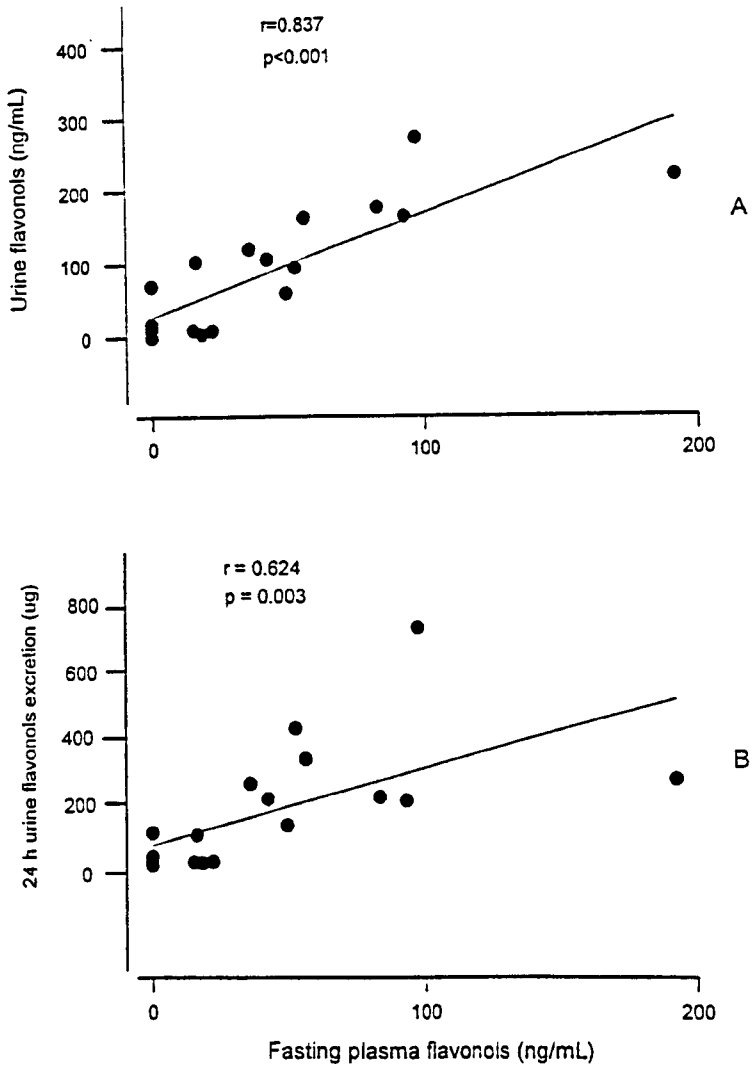


Figure 7.1 Relation between fasting plasma and urine concentration (A) or 24 *h* urine flavonoids excretion (B), studied on low and high flavonol diets in ten diabetic patients (NIDDM)

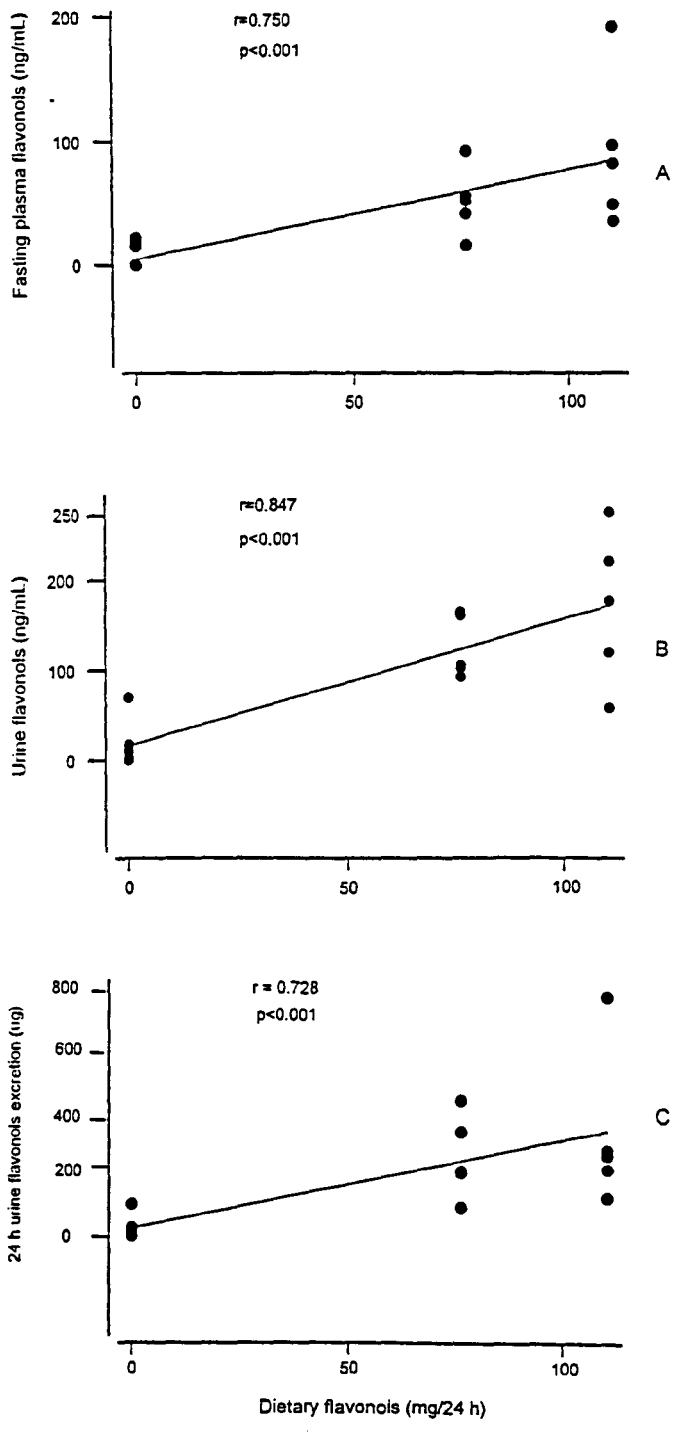


Figure 7.2 Dietary flavonol consumption superimposed on the regression lines for fasting plasma (A), urine concentration (B) or 24 *h* urine flavonol excretions (C).

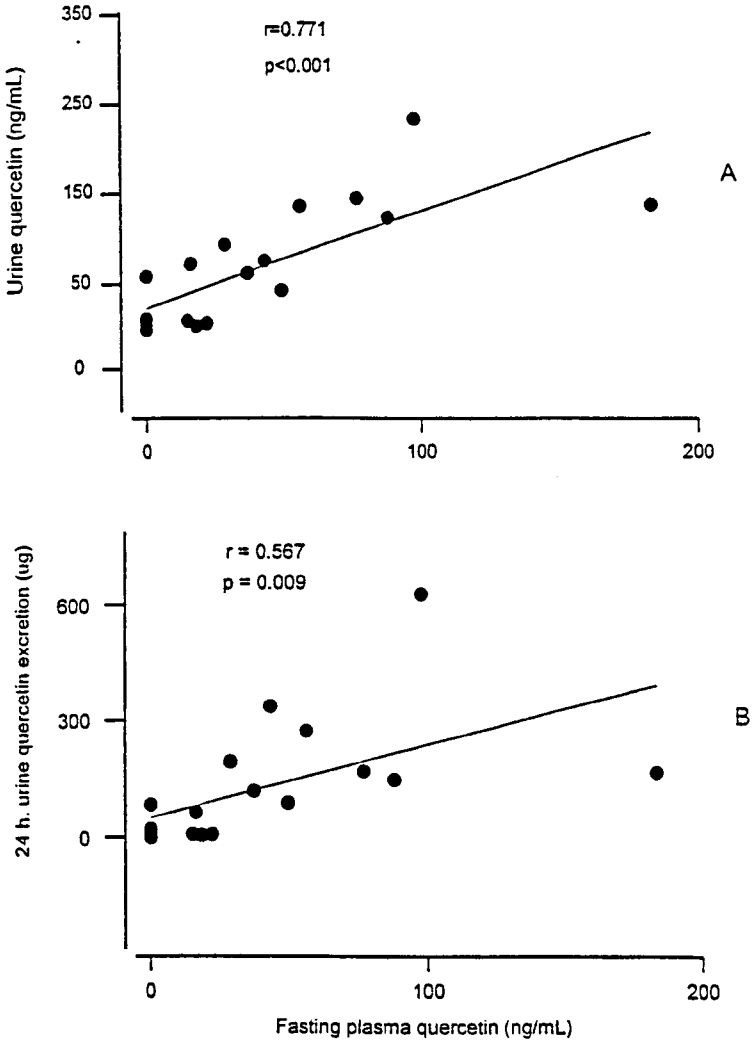


Figure 7.3 Relation between fasting plasma and urine concentration (A) or 24 *h* urine quercetin excretion (B), studied on low and high flavonol diets in ten diabetic patients

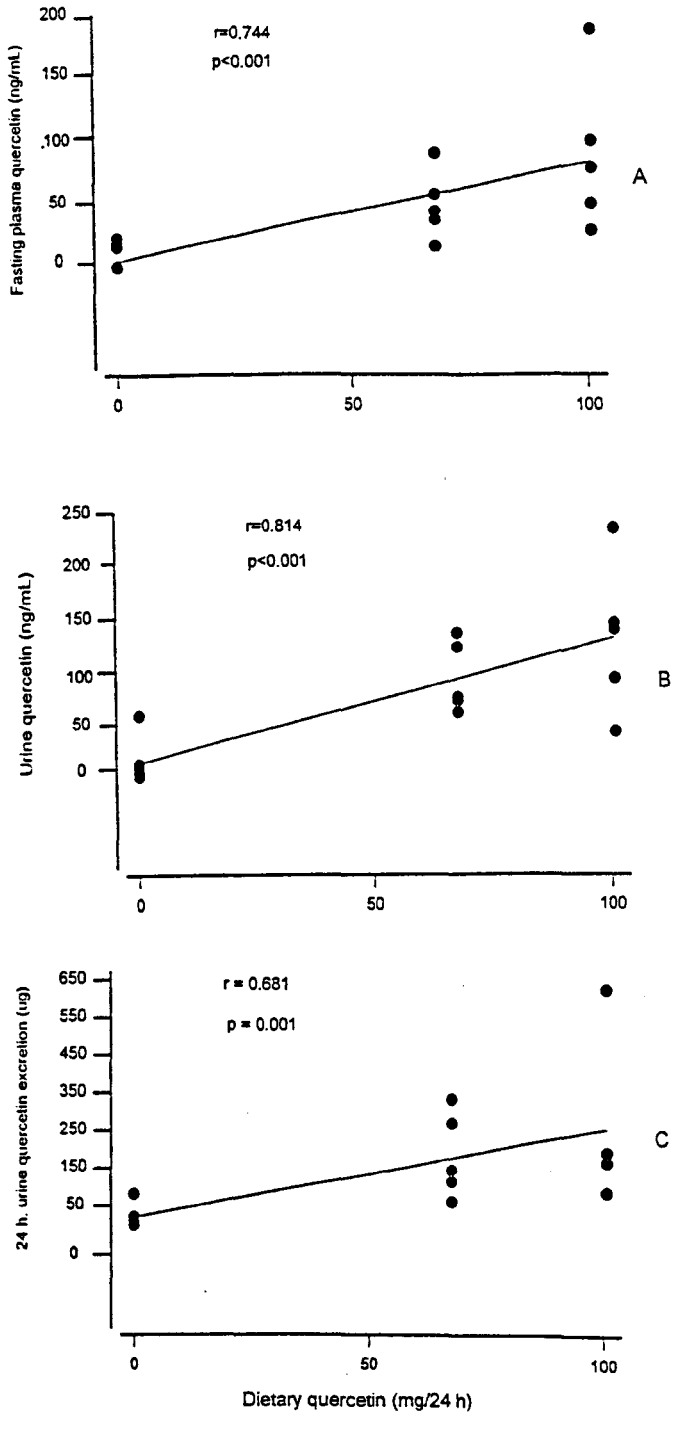


Figure 7.4 Dietary quercetin consumption superimposed on the regression lines for fasting plasma (A), urine concentration (B) or 24 *h* urine flavonol excretions (C).

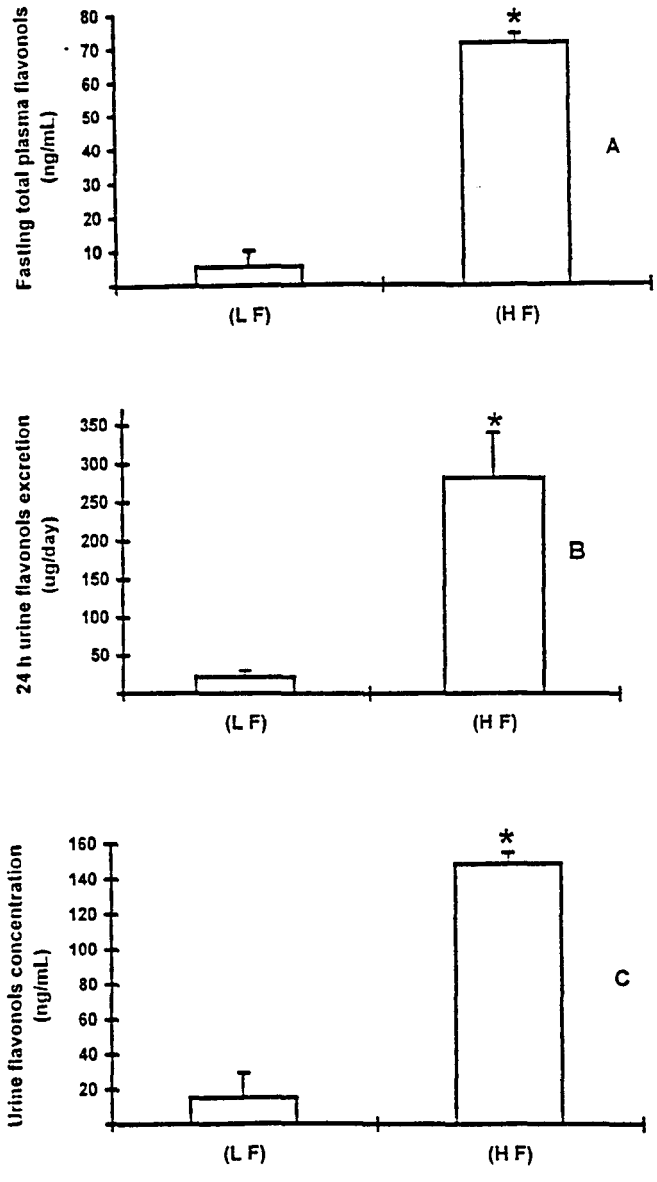


Figure 7.5 Plasma (A) and urine (B&C) flavonols of subjects (NIDDM) after low (LF) and high (HF) flavonol diet. Mean ± SEM, $n = 10$* $p<0.005$

Chapter 8: General conclusions

8.1 Answers to the research questions

8.2 Interpretations and recommendation for future research

The purpose of this chapter is to evaluate the extent to which this thesis has addressed the aims and answered the research questions which were outlined in Chapter 1, part 1.6).

Chapter 8.1 summarises and brings together the results of the various investigations within this thesis on aspects of antioxidant activity of flavonoids, specifically the major flavonols. An attempt is made at comparing antioxidant activity of flavonols with known antioxidants (e.g. vitamin C). This chapter also considers the biological effects of potential medical importance attributable to the absorption of dietary flavonols. Chapter 8.2 concludes with a brief note on the possible directions for future research in this field.

8.1 Answers to the research questions

8.1.1 Protection from various flavonoids and vitamin C against oxygen radical generated DNA damage in ex vivo lymphocytes in the SCGE or comet assay

Addressing Aim 1 and Aim 2, the work described in this thesis (Chapter 3) has explored the single cell gel electrophoresis (SCGE or comet assay) as a potential

tool for detecting the antioxidant effect of nutrients, and has shown reproducible results in estimating the extent of DNA damage to human lymphocytes to a standard challenge, and the degree of protection provided by pre-treatment with a range of flavonoids and vitamin C. It thus proved possible using this method to rank the potency of the antioxidant agents tested with high confidence.

All flavonoids tested in the comet assay demonstrated antioxidant capacity. Quercetin, myricetin and luteolin, with hydroxyl groups at the positions 3', 4' containing the unsaturated 2, 3-double bond in the C ring, were the most potent antioxidants. Luteolin, despite having a similar number of hydroxyl groups, was significantly more effective than kaempferol. This may be because the hydroxyl group at the 3' position (in the B ring) in luteolin confers greater antioxidant activity than the group at the 3 position (in the C ring) in kaempferol.

At equimolar concentrations the results demonstrate very clearly a greater antioxidant potency from most of the flavonoids tested than from vitamin C.

Research Question 1

The effects of quercetin, one of the most potently antioxidant flavonoids, and vitamin C, were additive when cells were pretreated with both at concentrations of 23.2 μmol/L in the comet assay. This does not necessarily imply that their actions would always be additive, if in fact they operate via the same

mechanisms. This finding does suggest that quercetin might provide a functional substitute when vitamins C status is low.

Research Question 2

This work extended the evidence that the position and number of hydroxyl groups have important roles in determining antioxidant activity. In our study, at a concentration of 279 μmol/l, the protection of myricetin, quercetin, kaempferol and apigenin against DNA damage would be consistent with a relationship to the number of hydroxyl groups.

Research Question 3

Aglycones quercetin, luteolin, myricetin and kaempferol had a greater antioxidative capacity than the conjugate flavonoids, such as quercetin-3-glucoside, quercitrin and rutin. Apigenin was the least potent of the free flavonoids. These results are in broad agreement with the other studies (Chapter 3, table 3).

The implications of this finding is that the flavonoids found in foods (almost all as conjugates) do not have the extreme potency of free flavonoids. It is too early to say how potent flavonoids are in the body, until more is known about their dispersal and metabolism. It does appear that some free flavonols may exist, at least in urine.

8.1.2 What are antioxidant activities of flavonoids and vitamin C in the trolox equipment antioxidant capacity (TEAC assay)?

As part of Aim 1, comet and TEAC assays were compared. All the flavonoids and polyphenols tested (except naringin) had greater antioxidant potency than vitamin C and several had greater capacity than Trolox itself, on a molar basis in the TEAC assay.

Greater numbers of hydroxyl groups in ring A, B or C of flavonoids and polyphenols significantly increased the TEAC. There was a positive relation between the number of hydroxyl groups of flavonoids and total antioxidant activity of flavonoids.

As with the comet assay, glycosylation of flavonoids consistently reduced the TEAC compared with aglycone flavonoids. Glycosylation of quercetin (at 3-hydroxyl group in the C ring) to form rutin (3-rutinoside), quercitrin (3-L-rhamnoside) and quercetin-3-glucoside decreases the antioxidant activity of these substances. These findings are of practical relevance since most flavonoids in foods occur as conjugates, not as aglycones.

A novel adaptation of the TEAC assay tested the putative antioxidants when added to fresh human plasma in supra-physiological concentrations (10, 20, 50, 100 μmol/l). The results support a physiological role for flavonoids in the body's antioxidant defence system. Quercetin proved the most potent, above

kaempferol and rutin at 100 μmol/l. Increases in the TEAC were observed when 50 or 100 μmol/l quercetin and 100 μmol/l rutin and kaempferol were added to the human plasma.

There has been particular interest recently in the antioxidant properties of tea. The results of TEAC on catechin, catechin esters (ECG and EGCG) and rutin indicate the antioxidant properties of these compounds which are found in teas. ECG had the greatest antioxidant activity of the 17 flavonoids and polyphenols tested, seven fold greater than vitamin C. These results were sufficiently persuasive to adopt tea as part of the later intervention study.

Research question 4

There was reasonable agreement between the comet and TEAC assays in the rank ordering of the 9 agents tested by both methods, although this failed to reach statistical significance (Spearman's rank correlation coefficient = 0.57, p =0.11). Both assays agreed in ranking quercetin the strongest and vitamin C the weakest antioxidant. However, luteolin and kaempferol, which were hightly ranked by the comet assay, appeared to have much weaker relative antioxidant activity by the TEAC assay, while the converse applied to rutin and quercetin-3-glucoside.

There are no strong a priori reasons for considering the comet or TEAC method to be more likely to be the "correct" one, and both gave reproducible results. It

seems reasonable to consider that the comet assay is more likely to relate to in vivo situations.

Luteolin was markedly more antioxidant in the comet assay than in the TEAC assay. One possible reason for differences between these methods is that the compounds tested may vary in lipid solubility. This would be expected to influence the comet assay by promoting uptake of more fat-soluble compounds into lymphocytes during the pre-treatment stage – much as might happen in the in vivo situations. Unfortunately it was not possible to test this by measuring the flavonoid content of lympocytes in the present studies.

8.1.3 Absorption of pure quercetin aglycone in humans

Addressing Aim 3, a major intervention study was undertaken. In the previous studies of this thesis, quercetin was found to be one of the strongest antioxidants, but the abosorption of quercetin in humans is unclear and unpredictable. Oral administation of quercetin dihydrate (aglycone) powder (1.8 and 6.2 mg/kg body weight) with different solvents (water or vodka 50% ethanol) did not change significantly the total antioxidant capacity of plasma (TEAC). There was some suggestion from one subject, after administration at 120 min showed a rise versus 0, 30, 60, 90, 180 and 240 minutes. However no detectable quercetin agylcone in plasma or in urine were found, and it was decided to proceed to a diet study. This was justified in that most flavonoids in normal diets are conjugated, and although the antioxidant capacity of conjugated forms of

flavonoids is less than aglycone (Chapter 3 and 4) they are present in high amounts of this form in the normal diet, and early evidence indicated greater absorption. It must be recognised that food sources of quercetin (e.g. onions and tea) are likely to contain other bioactive compounds, including other flavonoids such as catechins.

8.1.4 Form of flavonols in plasma and urine, and prediction of dietary flavonol consumption from fasting plasma concentration or urinary excretion

Chapter 7 focussed on the absorption and excretion of flavonols in humans, with the aim of establishing a biomarker for the flavonols intakes of free-living subjects. Onions and tea were chosen as the main foods, as important sources of quercetin although they do contain other potentially bioactive phenolics. Quercetin was the major flavonol in the test diets, and most flavonols in supplements were provided as conjugates. Flavonols in plasma were 100% conjugated, but in urine on baseline, low and high flavonoids diets the proportions conjugated were 82%, 67% and 87%. It cannot be ascertained from this study if free flavonols are more actively excreted in urine, or if deconjugation occurs as part of the excretion process.

Flavonols, mainly quercetin, were present in urine and plasma on the baseline diets of these subjects in every case. The dietary exposure was estimated from regression plots against intake on low flavonoid and one of two high flavonoid diets. Urinary and fasting plasma concentration of flavonols were highly

correlated and both values were highly significantly related to dietary intake. The average flavonol intake on the baseline diet estimated from fasting plasma flavonols was 35 mg/day, with a range 18-82 mg/day (32 mg/day quercetin). The subjects in this study (NIDDM) were not necessarily representative of the adult non diabetic population, but the results indicate that plasma or urine flavonols could be used to predict flavonols intake in future survey work.

8.1.5. Do dietary flavonols protect against oxidative DNA damage

Test diets based on onions and tea showed that the major flavonols were absorbable and the high flavonoids supplements, on the background of a low flavonoid diet, increased fasting flavonoid concentration approximately twelve-fold.

Diabetic patients, were chosen as a test group since they have increased free-radical production and also reduced antioxidant defences. Comet assay and endonucleuse III assay were used to attempt to identify and quantify oxidative DNA damage in the sugar phosphate backbone and in the bases of DNA.

High flavonoid diet designed based on onions and tea protected diabetic lymphocytes DNA against oxidative DNA damage from a standard H_2O_2 challenge and this effect was not induced by any change in known antioxidants including plamsa, vitamin C, tocopherol, carotenoids, urate, albumin, bilirubin.

The result was not related to fructosamine or fasting plasma glucose, so could not be ascribed to improved overall diabetic control.

This study has shown beneficial biological effect of flavonoids provided by a palatable test diet, it would seem appropriate to suggest the diets relatively high in flavonoids as well as other antioxidant vitamins should be recommended to protect diabetic patients against coronary complications. The evidence from the present study shows a potentially valuable effect of dietary flavonols in the range 76.3 – 110.4 mg/day, from diets containing about 500 g/day of fruit and vegetables and six cups of tea. Maintaining this level would offer a new benefit from achieving the existing recommendation of >400 g/day of fruits and vegetables. Onions are rich in flavonols, but other food combinations are possible.

8.2 Interpretations and recommendation for future research

The work in this thesis has extended knowledge in several theoretical and practical aspects of dietary flavonoids. However, a simple conclusion that flavonoids are beneficial cannot be drawn. There are many natural flavonoids, and some may have toxic effects at high concentrations or high intakes including the extracellular production of active oxygen species (in vitro) by dietary

flavonols (Canada et al 1990) or mutagenic activity of quercetin and related compounds (in vitro) (Bjeldanes et al 1977).

Bioactive compounds such as flavonoids, which are known to be absorbed and to have appropriate actions may be important factors for health which coexist in foods with the more familiar antioxidant vitamins, to which many health benefits have been attributed from dietary analyses. Flavonoid analysis in human diets is now becoming possible and the factors which govern their concentrations in foods are becoming better understood. Future work will need to consider which specific food sources to promote for health reasons recognising differences between strains.

The conclusions from these in vitro and ex vivo experiments that flavonoids may have important biological effects must remain tentative, until more is known about the absorption, distribution, metabolism and biological effects of flavonoids within the body. It is necessary to know that foods with absorbable flavonoids are being consumed, or the extent of absorption, and how food preparation, storage and cooking may affect composition. When this detailed information is available, a better indication of the health impact of flavonoids will be possible than can be derived from simplistic application of "food tables" of the total aglycone flavonoid contents.

From the work in this thesis, several future projects of interest can be identified:

1. Using SCGE assay and TEAC assay to quantify of antioxidant activity of more dietary flavonoids – such as epicatechins, gallate, catechin, silymarin, epigallocatechin gallate within the body (in tissues or body fluids rather than just in vitro), and their interactions with antioxidant vitamins.

2. Fate and metabolism of flavonoids in the body.

3 Epidemiological survey using plasma concentrations to explore relations between flavonoids consumption and diseases e.g. CHD, cancer, diabetes which involve free-radical-mediated processes.

4. The effect of high protein diet (milk, cheese, etc) or other dietary factors on absorption of flavonoids in human gut.

5. The effect of high flavonoids diet on absorption of iron and other elements in human diet.

5. The possible activity within the bowel of non-absorbed flavonoids when flavonoid intake is increased.

7. The impact of dietary flavonoids on free-radical mediated processes in diabetic patients – e.g. cataract, vascular disease, renal disease, glycosylation.

ACKNOWLEDGEMENTS

I am particularly indebted to Professor MEJ Lean, Head of Department of Human Nutrition, University of Glasgow in the Royal Infirmary for providing the facilities of the experiments, encouragement and kindly supportive role as a supervisor of the clinical and experimental aspects of this work.

I would like to thank Dr Andrew Collins (Rowett Research Institue, Aberdeen) for close support in training, for the comet assay and providing Endonuclease III enzyme. Thanks to Dr James Currall, for his patience in explaining statistics principles. Many thanks are due to Dr Wilson J Angerson: (Department of Surgery, Glasgow University, Royal Infirmary, Glasgow) for criticism and guidance, training for image analysis of comet assay, analysis of image and calibration of comet assay. He was a great source of reassurance.

Thanks to Dr Alan Crozier, J Burns and M McDonald (Institute of Biomedical and Life Sciences, Department of Plant Biochemistry and Molecular Biology, University of Glasgow) for guidance, collaboration and provide some flavonoids and for the vitally important analyses of flavonoids in foods, plasma and urine samples.

Thanks are due to all my colleagues at Glasgow Royal Infirmary Dr D Talwar, Dr H. Miller, Dr N. Sattar and Dr A Duncan, J Cooney, S Shanley (Institute of Biochemistry, Departments of Clinical Biochemistry , Glasgow Royal Infirmary)

for routine analyses conducted under the supervision of Dr Dinesh Talwar and Dr Naveed Sattar. Also Dr J Telfer and Dr H. Norman (Department of Obstetrics and Gynaecology) for collaboration with detection of SOD in plasma of NIDDM patients and Dr Koffi Aidoo (Caledonian University Department of Food Chemistry) for measurement of vitamins C and E in the test meal.

I would like to thank Irene Kelly, Dietitian in the Department of Human Nutrition, Glasgow Royal Infirmary for recruiting NIDDM subjects, completing and analyses of food composition tables and preparing test meals. Acknowledgements are also due to the NIDDM patients of the Diabetes Centre, Glasgow Royal Infirmary for their consent to take part in this research. Also thanks to Stewart Campbell and Kevin Walsh for volunteering to take part in the study and their kindly guidance.

Special thanks are due to Maureen McNee for editing and typing tables and text, her kindly guidance and patience.

This work was supported by the Ministry of Health and Medical Education of I.R. Iran.

Publication arising presentations

Full papers in refereed journals:

Noroozi M, Angerson WJ, Lean MEJ (1997). Effects of flavonoids and vitamin C on oxidative DNA damage to human lymphocytes. *American Journal of Clinical Nutrition.* (in press)

Lean MEJ, Noroozi M, Burns J, Talwar D, Sattar N, Crozier A (1998). Dietary flavonoids protect diabetic human lymphocytes against oxidative damage to DNA. Submitted to *Diabetes.*

Noroozi M, Miller H, Sattar N, Lean MEJ (1997). Antioxidant activities of vitamin C and flavonoids. Submitted to *American Journal of Clinical Nutrition.*

Noroozi M, Burns J, Crozier A, Kelly I, Lean MEJ (1998). Prediction of dietary flavonol intake from plasma and urinary flavonoid concentration in diabetic patients (NIDDM). To be submitted to *The American Journal of Clinical Nutrition.*

Noroozi M, Lean MEJ, Talwar D, Sattar N, Cooney J, Shanley S (1998). Association between fructosamine and antioxidants in diabetic patients (NIDDM). In preparation for *Diabetic Medicine.*

Abstracts:

Noroozi M, Angerson WJ, Collins A, Lean MEJ (1997). Protection from various flavonoids against oxygen-radical generated DNA damage in the single cell gel electrophoresis assay. *Proceedings of the Nutrition Society*. 56(106A).

Noroozi M, Burns J, Crozier A, Talwar D, Sattar N, Kelly IE, Lean MEJ (1998). Dietary flavonoids protect diabetic lymphocytes against oxidative DNA damage. *Diabetic Medicine*.

Presentations:

Protection from various flavonoids against oxygen-radical-generated DNA damage in the SCGE assay. The Nutrition Society meeting, University of Ulster, Northern Ireland, July 1996.

Antioxidant effects of flavonoids. University of Manchester (Umist) 3rd Iranian Medical Sciences meeting, December 1996.

Using comet assay for detection of antioxidant activity of three flavonoids (quercetin, kaempferol and quercetin-3-glucoside) for Dr Andrew Collins (Rowett Research Institute, Aberdeen) and co-workers in Glasgow Royal Infirmary, May 1996.

Studies with the comet assay. Glasgow University Flavonoid Group and The Antioxidant Group, Guy's Hospital, London. Glasgow, February 1998.

Dietary flavonoids protect diabetic lymphyocytes against oxidative DNA damage. British Diabetic Association, (BDA), Spring meeting Edinburgh, March 1998.

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